VOLUME 87
NUMBER 7 / NUMÉRO 7
JULY / JUILLET
2009

CABI BIOSCIENCE
EGHAM LIBRARY
-5 OCT 2009

# BOTANY

An International Journal for Plant Biology

SPECIAL ISSUE CELEBRATING THE 50TH ANNIVERSARY OF THE CANADIAN SOCIETY OF PLANT PHYSIOLOGISTS (CSPP)

botany.nrc.ca

# BOTANIQUE

Une revue internationale de biologie végétale

NUMÉRO SPÉCIAL DU 50E ANNIVERSAIRE DE LA SOCIÉTÉ CANADIENNE DE PHYSIOLOGIE VÉGÉTALE (SCPV)

botanique.cnrc.ca

An NRC Research Press
JOURNAL
Une REVUE des
Presses scientifiques du CNRC

50th ième

Annual Meeting of the Canadian Society of Plant Physiologists

Assemblée annuelle Société Canadienne de Physiologie Végétale

University of Ottawa
Université d'

June 14-17, 2008
14-17 juin 2008

National Research Council Canada
Conseil national de recherches Canada

NRC-CNRC

ISSN 1916-2790 / BOTAB3 87(7) 659–721 (2009)

## Notice to authors

**Manuscripts** for publication should be submitted to the editorial office via NRC Research Press' online submission and peer review system (**OSPR**EY) at http://pubs.nrc-cnrc.gc.ca/cjb/osprey. Alternatively, manuscripts may be submitted by mail to the Editors, **R. Larry Peterson** or **Dr. Barry J. Shelp**, *Botany*, Molecular and Cellular Biology, Science Complex, University of Guelph, Guelph, ON N1G 2W1, Canada (telephone: 519-824-4120 ext. 54597; fax: 519-827-9549; e-mail: canjbot2@uoguelph.ca). Authors may submit the names of persons capable of evaluating the scientific merit of their paper or, in cases of possible controversy, suggest who should not give such evaluation.

**Correspondence concerning accepted manuscripts and corrected proofs** should be sent to NRC Research Press, National Research Council of Canada, Ottawa, ON K1A 0R6, Canada.

**Instructions to Authors** are published at botany.nrc.ca. The **Publication Policy** of NRC Research Press is available at pubs.nrc-cnrc.gc.ca.

## Subscription information

*Botany* (ISSN 1916-2790) is published monthly by the National Research Council of Canada. The 2009 annual subscription rates (second-class postage) for Canada are $1230CAN institutional and $380CAN personal, for US and foreign $1230US institutional and $380US personal. For airmail add $180US to foreign rate. Canadian customers please include GST (business No. 12149 1807 RT 0275). Subscriptions are entered for the calendar year. Remittances should be made payable to the Receiver General for Canada, credit NRC. Price for regular single issues: institutional, $120; personal, $40. Supplements will be priced separately. Special rates apply to members of Canadian scientific and engineering societies if they subscribe through their respective societies.

All issues of *Botany* are available on the Web. Pricing information can be found at pubs.nrc-cnrc.gc.ca.

There are no refunds on cancelled subscriptions once delivery has begun. Claims for missing issues should be made within four months (North America) or six months (outside North America) following the date of publication. The publisher expects to supply missing issues only when losses have occurred in transit and when the reserve stock will permit. For change of address and subscription enquiries: Subscription Office, NRC Research Press, National Research Council of Canada, Ottawa, ON K1A 0R6, Canada; telephone: 613-993-9084; fax: 613-952-7656; e-mail: pubs@nrc-cnrc. gc.ca.

**Microform** backfiles are available from UMI, P.O. Box 1346, Ann Arbor, MI 48106-1346, USA, and Micromedia Ltd., 20 Victoria St., Toronto, ON M5C 2N8, Canada.

***Botany*, ISSN 1916-2790**, is published **Monthly, 12 times per year**, by **NRC Research Press c/o USACAN Media Dist. Srv. Corp.** at 26 Power Dam Way Suite S1-S3, Plattsburgh, NY 12901. Periodicals postage paid at Plattsburgh, NY and at additional mailing offices. POSTMASTER: Send address changes to ***Botany***, c/o NRC, P.O. BOX 2888, Plattsburgh, NY 12901.

## Note aux auteurs

Les auteurs sont invités à soumettre leur **manuscrit** au Bureau de la rédaction par le truchement du Système de soumission et d'examen en direct des articles (**OSPR**EY) des Presses scientifiques du CNRC à http://pubs.nrc-cnrc.gc.ca/cjb/osprey. Sinon, ils peuvent transmettre leur manuscrit par la poste aux rédacteurs en chef **R. Larry Peterson** ou **Barry J. Shelp**, *Botanique*, Molecular and Cellular Biology, Science Complex, University of Guelph, Guelph, ON N1G 2W1, Canada (tél : 519-824-4120, poste 54597; téléc. : 519-827-9549; courriel : canjbot2@uoguelph.ca). Les auteurs peuvent proposer les noms de personnes susceptibles de juger la valeur scientifique de leur manuscrit ou, s'il y a possibilité de controverse, d'indiquer les personnes qui ne devraient pas faire une telle évaluation.

La **correspondance ayant trait aux manuscrits acceptés** et les épreuves corrigées doivent être envoyées aux Presses scientifiques du CNRC, Conseil national de recherches du Canada, Ottawa, ON K1A 0R6, Canada.

Les **Recommandations aux auteurs** sont publiées au botanique. cnrc.ca. La *Politique éditoriale* est disponible au pubs.nrc-cnrc.gc.ca.

## Renseignements sur l'abonnement

*Botanique* (ISSN 1916-2790) est une publication mensuelle du Conseil national de recherches du Canada. Le prix de l'abonnement annuel de 2009 au Canada (courrier de deuxième classe) est de 1230 $CAN pour les organismes et de 380 $CAN pour les particuliers. À l'étranger (y compris les É.-U.), le prix de l'abonnement est de 1230 $US pour les organismes et de 380 $US pour les particuliers. L'expédition par avion exige des frais de 180 $US en sus du prix d'abonnement à l'étranger. Les clients du Canada doivent ajouter la TPS (numéro d'entreprise 12149 1807 RT 0275). Les abonnements sont pour l'année civile. Tout paiement doit être adressé au Receveur général du Canada, crédit du CNRC. Le prix par numéro est de 120 $ pour les organismes et de 40 $ pour les particuliers. Le prix des hors-série varie. Les membres des sociétés canadiennes de sciences et de génie bénéficient de taux préférentiels en s'abonnant via leurs sociétés respectives.

Tous les numéros *Botanique* sont accessibles sur le Web. On trouvera des renseignements sur les tarifs à pubs.nrc-cnrc.gc.ca.

Aucun remboursement ne sera accordé ni aucune annulation acceptée une fois que les livraisons auront commencé. Les réclamations de numéros manquants devront être soumises dans les quatre mois (Amérique du Nord) ou dans les six mois (à l'extérieur de l'Amérique du Nord) suivant la date de publication. L'éditeur ne fournira de numéros manquants que si le document a été perdu pendant l'expédition et que si le stock le permet. Adressez tout changement d'adresse ou toute question quant à l'abonnement au : Bureau des abonnements, Presses scientifiques du CNRC, Conseil national de recherches du Canada, Ottawa, ON K1A 0R6, Canada; tél. : 613- 993-9084; télec. : 613-952-7656; courriel : pubs@nrc-cnrc.gc.ca.

On peut obtenir les fichiers rétrospectifs sur **microforme** auprès de UMI, P.O. Box 1346, Ann Arbor, MI 48106-1346, É.-U., et auprès de Micromedia Ltée, 20, rue Victoria, Toronto, ON M5C 2N8, Canada.

**This journal is indexed or abstracted in / Cette revue est signalée ou résumée dans**
Agricola; BIOBASE; Biological & Agricultural Index; Biomedical Reference Collection; BIOSIS; CAB Abstracts; Chemical Abstracts; Crop Physiology Abstracts; Current Abstracts; Current Awareness in Biological Sciences; Current Contents; Derwent Biotechnology Abstracts; Field Crop Abstracts; PASCAL; Referativnyi Zhurnal; Science Citation Index

Volume 87, Number 7, July 2009

# Botany

Published by the National Research Council of Canada

Volume 87, numéro 7, juillet 2009

# Botanique

Publiée par le Conseil national de recherches du Canada

**Editors / Rédacteurs en chef** **R. Larry Peterson and Barry J. Shelp**

*Assistants to the Editors / Adjoints aux rédacteurs en chef* **Christopher Peterson, Wendy Gibbs**

**Associate Editors / Membres du Comité de rédaction**

J.F. Basinger (University of Saskatchewan)
S. Berch (B.C. Ministry of Forests)
P. Bonham-Smith (University of Saskachewan)
D.S. Coxson (University of Northern British Columbia)
Y. Dalpé (Agriculture and Agri-Food Canada, Ottawa)
A.R. Davis (University of Saskatchewan)
B. Downie (University of Kentucky)
G. Espie (University of Toronto at Mississauga)
P.J. Facchini (University of Calgary)
A. Fredeen (University of Northern British Columbia)
P. Goodwin (University of Guelph)
S. Graham (The University of British Columbia)
F. Guinel (Wilfrid Laurier University)
B. Husband (Univeristy of Guelph)
R. Ireland (Mount Allison University)
E. Johnson (University of Calgary)
M. Jones (Okanagan University College)
P.M. Kotanen (University of Toronto)
R. Latta (Dalhousie University)
R. Mullen (University of Guelph)
O.P. Rajora (University of New Brunswick)
D. Riggs (University of Toronto)
M.R. Roberts (University of New Brunswick)
S. Rood (University of Lethbridge)
P. Ryser (Laurentian University)
H.S. (Deep) Saini (University of Waterloo)
L. Samuels (The University of British Columbia)
H. Sanfaçon (Agriculture and Agri-Food Canada, Summerland Research Centre)
L. Sanità di Toppi (University of Parma)
G. Selvaraj (Plant Biotechnology Institute, National Research Council of Canada)
S.F. Shamoun (Canadian Forest Service, Pacific Forestry Centre)
A. Sherwood (University of Hawaii)
J. Starr (University of Ottawa)
S. Stefanović (University of Toronto at Mississauga)
G. Thorn (University of Western Ontario)
W. Untereiner (Brandon University)
V. Vandvik (University of Bergen)
L. Vasseur (Laurentian University)
D. Wolyn (University of Guelph)
T. Xing (Carleton University)

**NRC Research Press / Presses scientifiques du CNRC**

Fax / Télécopieur : 613-952-7656; e-mail / courriel : pubs@nrc-cnrc.gc.ca; URL / URL : pubs.nrc-cnrc.gc.ca

*Editor-in-Chief / Directeur de la publication* **Bruce P. Dancik**
*Deputy Editor-in-Chief / Sous-directrice de la publication* **Cindy E. Prescott**
*Publisher / Éditeur* **Cameron D. Macdonald**

*Business Manager / Gestionnaire des affaires commerciales* **Mike Boroczki**
*Advertising and reprints / Annonces publicitaires et tirés à part* **Roxanne Landriault** (613-990-2254)
*Subscriptions / Abonnements* **Eileen Evans-Nantais** (613-990-7873), **André Séguin** (613-993-9084)

*Manager, Journals Program / Gestionnaire du Programme des revues* **Carolyn Brown**
*Managing Editor / Directrice de la rédaction* **Cecily M. Pearson** (613-993-9099)
*Scientific Publishing Editor / Réviseure de publications scientifiques* **Jane Dormon**
*Editorial Publication Assistants / Adjoints à la publication* **Luc Julien, Diana Leck**
*Editorial Applications Training and Support Officer / Agente de formation et de soutien à l'éditique* **Mary Seligy**

*Production Manager / Gestionnaire de la production* **Brad Stoppa**
*Production Application Support Technician / Technicienne au soutien de la production* **Judy Hum**
*e-Publishing Technician / Technicienne à l'édition électronique* **Angela Lamarche**
*Graphics Technician / Technicien en graphisme* **Merv Buske**
*Web Technician / Technicien Web* **Dennis Cafferty**

The National Research Council of Canada accepts no responsibility for statements or opinions expressed by a contributor. Acceptance of an advertisement, announcement, or other material does not imply endorsement by either the Journal's Editors or the National Research Council of Canada.

**© 2009 National Research Council Canada or its licensors**

For articles published in 2009 and later, copyright remains with the authors, whereas for articles published before 2009, copyright is held by the National Research Council Canada.

No part of the NRC Research Press journals may be reproduced, stored, or transmitted in any form or by any means, without the written permission of the publisher, except as stated below.

Under the Canadian *Copyright Act*, individuals may download or print single copies of articles for personal research or study. Any person may reproduce short excerpts from articles in the journals for any purpose that respects the moral rights of authors, provided that the source is fully acknowledged. As a courtesy, the consent of authors of such material should be obtained directly from the author.

Authorization to reproduce items for other than personal research or study, as stated above, may be obtained via Access Copyright upon payment of the copyright fee of $10.00 per copy (Access Copyright, The Canadian Copyright Licensing Agency, 1 Yonge St., Suite 800, Toronto, ON M5E 1E5, Canada).

NRC Research Press also extends certain additional rights to authors.

The above rights do not extend to copying or reproduction for general distribution, for advertising or promotional purposes, for creating new collective works, or for resale. For such copying or reproduction, arrangements must be made with NRC Research Press (National Research Council Canada, Ottawa, ON K1A 0R6, Canada).

Le Conseil national de recherches du Canada ne se tient en aucune façon responsable des opinions exprimées par un collaborateur ou des déclarations que pourrait faire ce dernier. La publication d'annonces publicitaires, d'avis et autres n'implique pas l'endossement de leur contenu par les rédacteurs en chef de la Revue ou le Conseil national de recherches du Canada.

**© 2009 Presses scientifiques du CNRC ou ses concédants de licence**

Les auteurs détiennent les droits d'auteur sur les articles publiés en 2009 et plus tard, tandis que les Presses scientifiques du CNRC détiennent les droits d'auteurs sur les articles publiés avant 2009.

Il est interdit de reproduire, de sauvegarder ou de transmettre toute partie des revues des Presses scientifiques du CNRC dans quelque format ou de quelque façon que ce soit sans le consentement écrit de l'éditeur, sauf dans les cas énoncés ci-après.

Aux termes de la *Loi sur le droit d'auteur* du Canada, les particuliers peuvent télécharger ou imprimer des copies individuelles d'articles pour leurs études ou leurs recherches personnelles. Toute personne peut reproduire de courts extraits des articles d'une revue pourvu que les droits moraux de l'auteur soient respectés et que la source soit clairement indiquée. Il convient de demander directement à l'auteur son consentement.

On peut obtenir l'autorisation de reproduire des articles à d'autres fins que les fins d'études et de recherches personnelles susmentionnées, et ce, par l'intermédiaire d'Access Copyright, moyennant des redevances de droits d'auteur de 10 $ par copie (Access Copyright, The Canadian Copyright Licensing Agency, 1, rue Yonge, bureau 800, Toronto, ON M5E 1E5, Canada).

Les Presses scientifiques du CNRC accordent aussi des privilèges supplémentaires aux auteurs.

Les droits ci-dessus ne s'appliquent pas à la reproduction à grande échelle aux fins de distribution générale, de publicité ou de promotion, de compilation de nouveaux ouvrages collectifs ou de revente, par exemple. Les droits de reproduction de ce genre doivent faire l'objet d'une entente particulière avec les Presses scientifiques du CNRC (Conseil national de recherches du Canada, Ottawa, ON K1A 0R6, Canada).

**Special Issue celebrating the 50th Anniversary of the Canadian Society of Plant Physiologists (CSPP)**

**Numéro spécial du 50e anniversaire de la Société canadienne de physiologie végétale (SCPV)**

# INTRODUCTION

## Introduction to the Special Issue celebrating the 50th anniversary of the Canadian Society of Plant Physiologists (CSPP)

The peer-reviewed papers in this special issue are based on contributions to the annual meeting of the Canadian Society of Plant Physiologists (CSPP), which was held in Ottawa in 2008 and celebrated the 50th anniversary for the organization. The Society is grateful for the work of the local organizing committee including, John T. Arnason (co-chair) and Brian Miki (co-chair), Christiane Charest, Douglas A. Johnson, Susan Aitken, Tim Xing, and Leo Savitch, who took on the task of organizing the scientific meeting and the 50th anniversary celebration. The organizing committee included members from the University of Ottawa, Carleton University, and Agriculture and Agri-Food Canada (Ottawa), and was assisted by 26 student volunteers.

Over 320 people attended the meeting and participated in a program that included 18 plenary speakers, 32 speakers at concurrent sessions, and 150 posters. Important for the health of the Society, this included a good mix of emeritus members, long-term members, and over 100 students.

Many excellent talks were given on recent developments in plant science and the critical role that the knowledge plays in solving some of the major issues for our planet, namely the security of our food supply, meeting our energy needs, and the global environment. As is the nature of the current Society, the scientific program presented was an interesting mix of whole plant, cellular, and molecular physiology. The sessions were inspiring and thought provoking.

The co-editors of *Botany* (Dr. Barry J. Shelp and Dr. R. Larry Peterson) worked with the local organizing committee of the meeting, who served as special editors for this issue, to publish a cross-section of the interesting science presented at the meeting. This is an important collection for plant science in Canada because it provides a snapshot in time of the excellent level of science that was discussed at the meeting and gives insight into issues that are currently driving plant science research in Canada and the world.

Because it was an anniversary, a look back to the founding of the Society was important. Connie Nozzolillo provided an excellent overview of the people who were instrumental in organizing plant science meetings in Canada, which led to the formation of the CSPP. The annual meeting was also an occasion to celebrate the completion of Connie's chronicle on the history of the CSPP entitled *The Second Twenty-Five Years*, which was provided to attendees of the meeting. It is wonderful to see where we have been, know who was involved and have an appreciation of the forces that shaped the Society we now enjoy.

The success of the meeting, reflected in the excellent articles in this special edition of *Botany*, gives us every confidence that the Society will continue to be a vibrant force in plant science in Canada.

**K. Peter Pauls**
*President of CSPP*

Digitized by the Internet Archive
in 2025

https://archive.org/details/botanisches-centralblatt_1916_131_25

# INTRODUCTION

## Introduction du numéro spécial du 50e anniversaire de la Société canadienne de physiologie végétale (SCPV)

Les articles évalués par des pairs de ce numéro spécial sont tirés de communications présentées à la réunion annuelle de la Société canadienne de physiologie végétale (SCPV), tenue à Ottawa en 2008, où on a célébré le 50e anniversaire de l'organisme. La Société est reconnaissante du travail des membres du comité organisateur local, dont John T. Arnason et Brian Miki (coprésidents), Christiane Charest, Douglas A. Johnson, Susan Aitken, Tim Xing, et Leo Savitch, qui ont entrepris la tâche d'organiser la réunion scientifique et les célébrations du 50e anniversaire. Le comité organisateur regroupait des membres de l'Université d'Ottawa, de l'Université Carleton et d'Agriculture et Agroalimentaire Canada (Ottawa), aidés de 26 étudiants bénévoles.

Plus de 320 personnes ont assisté à la réunion et ont pris part à un programme qui comptait 18 conférenciers aux séances plénières, 32 conférenciers aux séances simultanées et 150 affiches. Cette réunion a regroupé tout un éventail de participants dont des membres émérites, des membres de longue date et plus de 100 étudiants, ce qui est important pour la santé de la Société.

Un grand nombre d'exposés remarquables ont été donnés sur l'évolution récente des sciences végétales et le rôle crucial que jouent les connaissances dans la résolution de certains problèmes importants pour la planète, à savoir la sécurité de l'approvisionnement alimentaire, la satisfaction des besoins énergétiques et l'environnement. Tout comme la nature même de la Société actuelle, le programme scientifique présenté comportait un éventail intéressant de sujets touchant la physiologie moléculaire, cellulaire et de la plante entière. Les séances ont été inspirantes et ont suscité la réflexion.

Les corédacteurs en chef de *Botanique* (Barry J. Shelp et R. Larry Peterson) ont travaillé avec le comité organisateur local de la réunion, qui a servi de comité de rédaction pour ce numéro spécial, afin de publier une vue transversale des sujets d'intérêt présentés à la réunion. Il s'agit d'un recueil important pour les sciences végétales au Canada, car il donne un aperçu de l'excellence du calibre des sujets scientifiques abordés au cours de la réunion et permet de mieux comprendre les questions qui stimulent actuellement la recherche en sciences végétales au Canada et ailleurs dans le monde.

Comme cette réunion soulignait un anniversaire, un retour en arrière sur la fondation de la Société s'avérait important. Connie Nozzolillo a présenté une excellente vue d'ensemble des gens qui ont contribué à l'organisation des réunions en sciences végétales au Canada, ce qui a mené à la naissance de la SCPV. De plus, cette réunion annuelle a été l'occasion de célébrer la réalisation, par Connie, d'une chronique sur l'histoire de la SCPV intitulée « *The Second Twenty-Five Years* », laquelle a été distribuée aux personnes présentes à la réunion. Il est formidable de voir le chemin parcouru, de savoir quelles personnes y ont joué un rôle et de comprendre les forces qui ont façonné la Société telle que nous la connaissons aujourd'hui.

Le succès de cette réunion, illustré par les articles remarquables que contient ce numéro spécial de *Botanique*, nous convainc, sans l'ombre d'un doute, que la Société continuera de représenter une force dynamique au sein des sciences végétales au Canada.

**K. Peter Pauls**
*Président de la SCPV*

# INTRODUCTION

## Introduction to "Nitrate uptake by plant roots" by Anthony Glass

The peer-reviewed lead article in this issue was invited to honour Dr. Anthony Glass, Department of Botany, University of British Columbia, Vancouver, British Columbia, as the recipient of the 2007 Gold Medal from the Canadian Society of Plant Physiologists.[1]

Dr. Glass is recognized for his outstanding contributions to the characterization of nitrate and ammonium transporters in plants and the identification of genes that encode these transporters. In the Award presentation, it was emphasized that his research involves physiological and biochemical approaches, as well as molecular biology and genomics, and that it is of both fundamental and practical significance.

Dr. Glass and his group were the first to use short-lived radiotracers ($^{13}NO_3^-$ and $^{13}NH_4^+$) to identify and characterize five distinct root nitrogen transporters by their kinetic properties (i.e., high versus low affinity, and constitutive versus inducible transport) in barley, rice, and tree species. These studies were complemented by thermodynamic descriptions, using electrophysiological methods. More recently, he cloned genes encoding the nitrogen transporters, studied their regulation, gene structure, and functional relations. Now, he is applying current concepts of transport processes to real problems in agriculture in collaboration with scientists at Agriculture and Agri-Food Canada and growers at the BC Hydroponic Growers Cooperative.

Professor Glass has published approximately 145 peer-reviewed papers in leading international journals such as *Trends in Plant Science*, *Plant Physiology*, *Proceedings of the National Academy of Sciences of the United States of America*, *Nature*, *Plant Journal*, *EMBO Journal*, *Journal of Biological Chemistry*, *Biochemistry*, and *Plant Molecular Biology*. He has also published seven book chapters and one textbook. He is the author or co-author of about 10 papers that have each been cited more than 100 times, resulting in his inclusion on the 2006 ISI Ranking of scientists "Most Highly Cited"; individuals in this group "are the most highly cited within each category for the period 1981 to 1999 and constitute less than a half-percent of all publishing researchers". Also, Dr. Glass has served on editorial boards for *Plant Physiology*, the *Canadian Journal of Botany* (now *Botany*), *Tree Physiology*, and *Acta Botanica*, and is currently on the editorial board for *Plant Form and Function*.

Dr. Glass has recruited, trained, and collaborated with an impressive group of very talented research personnel and has established local, national, and international collaborations. His talents for focusing on important problems and generating new insights based on data from his laboratory have resulted in a well-deserved international reputation in the field of nutrient transport.

We take this opportunity to congratulate Dr. Glass for this award that recognizes his distinguished career.

**Barry Shelp**
*Co-Editor*

**R. Larry Peterson FRSC**
*Co-Editor*

[1] A version of this was previously published in the Bulletin of the Canadian Society of Plant Pathologists, Issue 11, October 2007.

# INTRODUCTION

## Introduction pour « Nitrate uptake by plant roots » [Absorption du nitrate par les racines des plantes] d'Anthony Glass

L'article vedette du présent numéro a été sollicité pour saluer Anthony Glass du Département de botanique de l'Université de la Colombie-Britannique, Vancouver (Colombie-Britannique), lauréat de la Médaille d'or (2007) de la Société canadienne de physiologie végétale.[1]

M. Glass est applaudi pour sa contribution exceptionnelle à la caractérisation des transporteurs de nitrate et d'ammonium dans les plantes et à l'identification des gènes qui codent ces transporteurs. À la remise de la médaille, on a souligné que sa recherche met à profit des connaissances de la physiologie, de la biochimie, de la biologie moléculaire et de la génomique, ce qui est de grand intérêt sur le plan fondamental et pratique.

M. Glass et son groupe ont été les premiers à utiliser des indicateurs radioactifs transitoires ($^{13}NO_3^-$ et $^{13}NH_4^+$) pour localiser et caractériser cinq groupes distincts de transporteurs d'azote racinaires par leurs propriétés cinétiques (c'est-à-dire affinité élevée ou faible et transport constitutif ou inductif) dans l'orge, le riz et des espèces arborescentes. À ces études se sont ajoutées des analyses thermodynamiques, réalisées à l'aide de méthodes électrophysiologiques. Plus récemment, il a cloné les gènes qui codent des transporteurs d'azote ainsi qu'étudié leur régulation, leur structure génique et leurs relations fonctionnelles. Il applique maintenant les concepts associés aux processus de transport à des problèmes dans le domaine de l'agriculture en collaboration avec des chercheurs d'Agriculture et Agroalimentaire Canada et des cultivateurs de la BC Hydroponic Growers Cooperative.

M. Glass a signé près de 145 articles scientifiques soumis à l'examen par des pairs. Ces articles ont paru dans des revues internationales comme *Trends in Plant Science*, *Plant Physiology*, *Proceedings of the National Academy of Sciences of the United States of America*, *Nature*, *Plant Journal*, *EMBO Journal*, *Journal of Biological Chemistry*, *Biochemistry* et *Plant Molecular Biology*. Il a également publié sept chapitres de livres et un manuel. Il est auteur ou coauteur d'une dizaine d'articles qui ont été cités plus de 100 fois, ce qui lui a permis de figurer parmi les auteurs les plus cités, selon le Journal Citation Report de 2006 de Thomson ISI. Ces auteurs sont les plus cités dans chacune des catégories pour la période de 1981 à 1999 et représentent moins d'un demi-pour cent de tous les chercheurs-auteurs. De plus, M. Glass a siégé au comité de rédaction de *Plant Physiology*, de la *Revue canadienne de botanique* (aujourd'hui *Botanique*), de *Tree Physiology* et d'*Acta Botanica*, et fait actuellement partie du comité de rédaction de *Plant Form and Function*.

M. Glass a recruté et formé un groupe impressionnant de chercheurs extrêmement talentueux et établi des collaborations fructueuses à l'échelle locale, nationale et internationale. Ses dons naturels pour cibler les problèmes d'importance et générer de nouvelles idées issues de ses résultats de recherche lui ont valu une réputation internationale bien méritée dans le domaine du transport des éléments nutritifs.

Nous tenons à profiter de l'occasion pour féliciter M. Glass pour le prix qui lui a été décerné en reconnaissance de sa brillante carrière.

**Barry Shelp**
*Corédacteur en chef*

**R. Larry Peterson, MSRC**
*Corédacteur en chef*

[1] Des extraits de la version originale de cette introduction proviennent du bulletin d'automne (2007) de la Société canadienne de physiologie végétale.

MINIREVIEW / MINISYNTHÈSE

# Nitrate uptake by plant roots[1]

**Anthony D.M. Glass**

**Abstract:** Nitrate is a major source of N for plant growth, particularly under aerobic agricultural conditions. This nutrient is absorbed from soil solution by both high-affinity and low-affinity transport systems that were first defined physiologically and subsequently by molecular genetic means. This minireview presents an abbreviated history of developments in plant inorganic ion uptake, the physiology of nitrate uptake, and recent developments in the molecular genetic characterization of nitrate transport systems.

**Résumé :** Le nitrate constitue la source majeure de N pour la croissance des plantes, surtout sous les conditions agricoles aérobies. Ce nutriment est absorbé à partir de la solution de sol, à la fois par des systèmes de transport à haute et à faible affinités qu'on a d'abord définis physiologiquement et subséquemment au moyen de la génétique moléculaire. Dans cette revue, l'auteur présente une courte histoire de la mise en évidence de l'absorption organique des ions chez les plantes, de la physiologie de l'absorption des nitrates, ainsi que des derniers développements de la caractérisation par génétique moléculaire des systèmes de transport des nitrates.

[Traduit par la Rédaction]

## General introduction and historical outline

The study of nutrient uptake by plant roots has a long history. Despite the fact that de Sausure had demonstrated selective uptake of solutes as early as 1804, in 1840 the renowned German Chemist Liebig wrote that "All substances in solution in a soil are absorbed by the roots of plants, exactly as a sponge imbibes a liquid and all that it contains, without selection" (Epstein 1972). Our knowledge of ion uptake has come a long way in the more than 150 years since Liebig's pronouncement. During this time, ion transport has been investigated using many different methodologies that have established a number of fundamental characteristics of the process. These include (*i*) the concept of metabolic dependence, (*ii*) the enzyme-like kinetics of transport, (*iii*) the electrophysiological and biochemical aspects of ion transport, (*iv*) the dynamic nature of ion fluxes that adjust to meet whole plant demand, and most recently, (*v*) the molecular/genetic characterization.

Ironically, the proposition, based upon the ease of permeation of lipid-soluble molecules, that the cell membrane was composed of lipid (Overton 1895) led to the erroneous belief that the rate of entry of solutes into plant cells was a function of two criteria: membrane permeability and diffusion pressure (i.e., concentration gradient). This is evident in a series of review articles by Stiles (1924). Hoagland put this misconception to rest when he demonstrated that bromide accumulation by the alga *Nitella* was a function of light intensity; virtually no accumulation occurred in darkness (Hoagland et al. 1926). Also, internal concentrations of various naturally occurring ions were considerably higher than those of the media in which they were growing. For the cations this might have been explained by the influence of the negatively charged electrical potential difference, but for chloride this was impossible to explain by "diffusion pressure." Hoagland also showed that ion uptake by barley roots was a function of available oxygen tension (Hoagland and Broyer 1936). This established the intimate relationship between metabolic processes and solute uptake. A major development in the 1950s was the enzyme kinetic model whereby solute entry across root cell membranes was postulated to depend upon solute interaction with membrane-localized (enzyme-like) carriers (Epstein 1976). This work built upon the earlier demonstration by van den Honert that phosphate uptake by sugarcane roots took the form of rectangular hyperbolae when plotted against external phosphate concentration (van den Honert 1937). The observed saturation was interpreted as signifying the presence of a finite number of membrane structures responsible for absorbing phosphate. This saturation was demonstrated for nitrate uptake by *Zea mays* L. roots (van den Honert and Hooymans 1955). Epstein demonstrated the specificity of the carriers and showed that they were of two kinds, namely, high-affinity transport systems (HATS) operating at a low external concentration, and low-affinity transport systems (LATS) operating at a high external concentration. The use of electrophysiological methods established the existence of large electrical potential differences across plant cell membranes, and enabled active transport

Received 6 January 2009. Published on the NRC Research Press Web site at botany.nrc.ca on 10 July 2009.

**A.D. Glass.** Department of Botany, University of British Columbia, 6270 University Boulevard, Vancouver, BC V6T1Z4, Canada (e-mail: aglass@interchange.ubc.ca).

[1]This paper is one of a selection published in a Special Issue comprising papers presented at the 50th Annual Meeting of the Canadian Society of Plant Physiologists (CSPP) held at the University of Ottawa, Ontario, in June 2008.

 doi:10.1139/B09-014 

to be evaluated on solid thermodynamic criteria. Prior to this, various studies made use of metabolic inhibitors to investigate active transport, erroneously concluding that active transport was underway whenever rates of ion uptake were reduced by the inhibitor. The error here is that even passive transport driven by an electrical potential difference ($\Delta\varphi$) would show a reduction because of the metabolic dependence of $\Delta\varphi$. Using the electrophysiological methodology, Higinbotham et al. (1967) concluded that all anions, including nitrate, were actively transported into roots of barley and pea. These two approaches, kinetic and electrophysiological, should have been complementary, but their advocates tended to reside in separate solitudes. Soon after the discovery of membrane-localized Na/K ATPases in animals in the 1950s, plant studies established that plants too possessed plasma membrane-localized ATPases. Of these, perhaps the most important turned out to be outwardly directed proton ATPases (Poole 1978). These generate pH and electrical gradients across the cell membrane that serve as the energy source to drive many secondary transport reactions such as nitrate uptake. The general acceptance of Peter Mitchell's Chemiosmotic Hypothesis allowed the findings concerning plant membrane proton ATPases to be absorbed into the more broadly based Chemiosmotic Hypothesis of membrane transport. Many of the earlier studies of nutrient uptake tended to view ion transport strictly as a localized membrane process divorced from the whole plant context, and as a consequence, models of ion transport failed to recognize the dynamic and interactive nature of the process. The work of a number of scientists in the 1970s emphasized that ion fluxes are plastic, adjusting rates of nutrient uptake to match plant demand dictated by rates of growth. This allows for the maintenance of internal "set-points," which are presumably dictated by physiological/metabolic requirements for these ions (Cram 1976). For example, cytoplasmic $K^+$ concentration is considered to be maintained at $\sim$150 mmol·L$^{-1}$ to sustain protein synthesis (Wyn Jones et al. 1979). Beginning in the 1980s, we have witnessed the application of molecular genetics to ion transport and even a return to the biochemical methodology as physiologists attempt to characterize the proteins identified by molecular genetic techniques. Currently, the primary emphasis in studies of ion transport across plant membranes is on these molecular and biochemical methods.

## Nitrogen uptake

Because of its primary role as an obligate constituent of proteins, nucleic acids, and many secondary products, plants typically contain up to 4% of their dry weight in the form of nitrogen (N). This N is obtained from soils or aquatic systems in the form of nitrate ($NO_3^-$), ammonium ($NH_4^+$), or amino acids. Nevertheless, many reviews of this topic have commenced by stating that $NO_3^-$ is the "preferred" form of N for plants. Not only have such statements been criticized for anthropomorphy, but more importantly they ignore vast areas of forest lands, as well as rice paddies, where $NO_3^-$ is undetectable and $NH_4^+$ or amino acids predominate (Stark and Hart 1997). Likewise, in artic tundra, $NO_3^-$ is generally unavailable and the combined amino acid concentration may even exceed that of $NH_4^+$ (Kielland 1994). Not surprisingly, rates of $NH_4^+$ or amino acid uptake by plants that are native to such habitats may be significantly higher than corresponding rates of $NO_3^-$ uptake, especially when both forms of N are present simultaneously (Kielland 1994; Kronzucker et al. 1997). Indeed, even plants that normally grow in regions where $NO_3^-$ predominates still typically take up $NH_4^+$ at higher rates than $NO_3^-$, when both forms are present. For example, in some forests where available $NO_3^-$ concentrations greatly exceed those of $NH_4^+$, $NO_3^-$ uptake is blocked by the inhibitory effect of the presence of $NH_4^+$ (Gessler et al. 1998). Yet, most of the many studies of N uptake by plants have focussed on $NO_3^-$, to a lesser extent on $NH_4^+$ and to an even lesser extent on amino acids and $NO_2^-$. For example, searching Biosis Previews with the terms nitrate, nitrite, ammonium or amino acid uptake and limiting the search to plants gave 1402 papers for nitrate uptake, 71 papers for nitrite uptake, 538 for ammonium uptake and 391 papers for amino acid uptake.

In aerobic soils, especially agricultural soils (except for rice paddies) $NO_3^-$ is the predominant form in which N is absorbed, despite the fact that the bulk of N fertilizer is applied in the form of urea or ammonia, because of the rapidity of nitrification. In colder or water-logged soils, nitrite ($NO_2^-$) may accumulate in significant quantities (Lee 1979). Also, when plant $NO_3^-$ uptake exceeds the capacity to convert the $NO_2^-$ produced to $NH_4^+$, a large part of absorbed $NO_3^-$ may be released to the external medium as $NO_2^-$ (Needoba et al. 2004). The reason for this is that the reduction of $NO_3^-$ to $NO_2^-$, a two-electron step, may be achieved through use of anaerobically generated NADH. However, the next steps in assimilating $NO_2^-$ to the level of amino acids are demanding energetically (a six-electron reduction step to convert $NO_2^-$ to $NH_4^+$) and in terms of required carbon skeletons for the assimilation of ammonium into glutamine. Microrganisms, fungi and green plants all appear to have plasma membrane nitrate/nitrite bispecific transporters, as well as nitrite-specific transporters (Galván et al. 1996; Galván and Fernández 2001; Wang et al. 2007; Z. Kotur and A.D.M. Glass, unpublished data) in addition to chloroplast-membrane $NO_2^-$ transporters (Mariscal et al. 2004). Therefore $NO_2^-$ released to the environment in excess of assimilatory capacity may readily be absorbed later by nitrate/nitrite transporters or by nitrite-specific transporters.

Much of the earlier physiological work on nitrate uptake by plants has already been extensively reviewed (e.g., Glass and Siddiqi 1995); therefore, only key points are summarized in this review. Since the molecular studies dealing with nitrate uptake up to 2000 have been comprehensively reviewed (Forde 2000), here I focus mainly on subsequent developments.

### General characteristics of nitrate uptake

Three characteristics of $NO_3^-$ uptake were established early on: (*i*) Rates of net $NO_3^-$ uptake by seedling plants after several days growth without an exogenous $NO_3^-$ source may be too low to be accurately measured by net nitrate depletion but increase after exposure to $NO_3^-$ (Jackson et al. 1972). Because of the relatively small depletion, measuring uptake at high external concentration by net $^{14}NO_3^-$ depletion is problematic and may require long uptake periods. Measurements of net $NO_3^-$ uptake also suffer the problem that they represent the balance between influx and efflux.

Hence, more recent studies have seen increasing use of $^{13}NO_3^-$ or $^{15}NO_3^-$ to measure influx or efflux. After exposure to $NO_3^-$ for 3 to 6 h, $NO_3^-$ uptake rates are substantially increased (this process is termed induction); only $NO_3^-$ itself is capable of inducing $NO_3^-$ uptake. This induction is unique to $NO_3^-$ and is not the case for other ions such as $NH_4^+$ or $K^+$ when plants are grown without these nutrients. Rather the opposite is true; uptake rates of these and most other ions are initially exceedingly high and decline as internal concentrations build; (*ii*) Plots of $NO_3^-$ uptake by $NO_3^-$-induced plant roots versus external concentration give rectangular hyperbolae at low concentrations (up to ~0.1 to 0.2 mmol·L$^{-1}$), but at higher concentrations give linear plots, (van den Honert and Hooymans 1955; Doddema and Telkamp 1979; Pace and McClure 1986; Siddiqi et al. 1990; Guy and Heimer 1993). These are the two transport systems described above as HATS and LATS, respectively. It should be noted here that a seminal paper on $NO_3^-$ uptake by *Arabidopsis thaliana* (L.) Heynh reported by Doddema and Telkamp (1979) presented their HATS and LATS data on a log scale, erroneously interpreting their LATS data as conforming to a rectangular hyperbola for which $K_m$ and $V_{max}$ values were calculated. Linear plots give much higher $r^2$ values for regression. Even some contemporary studies continue to propagate this error, presuming hyperbolae without testing for linearity; (*iii*) The presence of $NH_4^+$ in external media causes a rapid and reversible reduction of $NO_3^-$ uptake (Minotti et al. 1969).

### Three physiologically defined nitrate transport systems

The use of more sensitive techniques for measuring net $NO_3^-$ uptake (e.g., HPLC) or unidirectional $NO_3^-$ influx by means of $^{13}NO_3^-$ revealed the presence of a saturable high-affinity transport system in barley roots even prior to exposure to $NO_3^-$ (Behl et al. 1988; Siddiqi et al. 1990; Aslam et al. 1992). This was named the constitutive high-affinity transport system (CHATS). This CHATS has been demonstrated to be present in other species such as white spruce and *A. thaliana* (Kronzucker et al. 1995; Wang and Crawford 1996). Generally, maximum $NO_3^-$ fluxes generated by CHATS are small compared with fluxes measured after induction by $NO_3^-$. For example, in barley roots the $V_{max}$ for $^{13}NO_3^-$ influx increased 30-fold from 0.3 μmol·g(FW)$^{-1}$·h$^{-1}$ in uninduced roots to 9 μmol·g(FW)$^{-1}$·h$^{-1}$ after exposure to $NO_3^-$ (Siddiqi et al. 1990). Yet in 'Steptoe' barley (King et al. 1993) and in *A. thaliana* (Okamoto et al. 2003) CHATS may be ~20% of the fully induced flux. Interestingly, although the term constitutive aptly describes this "system in waiting," it appears from studies using seedlings of barley and white spruce that exposure to nitrate causes the CHATS to be increased by about 50% (Aslam et al. 1992; Kronzucker et al. 1995). The discovery of the CHATS provided an explanation for an otherwise problematic issue concerning the so-called "induction" by $NO_3^-$; namely, how did the $NO_3^-$ get into the root to induce increased transport? Alternatively, induction might have been mediated by external nitrate initiating a second messenger system without actually entering root cells. The presence of this "in waiting" system provides for entry and amplification of transport. Nevertheless, an explanation for nitrate's uniqueness regarding induction is as yet unavailable. The enhanced HATS after induction is referred to as inducible HATS (IHATS). Lineweaver Burke and Hoffstee plots of the low-concentration $NO_3^-$ fluxes in roots of induced plants are bimodal demonstrating that both the CHATS and the IHATS are simultaneously expressed, rather than the IHATS replacing the CHATS or representing an expansion of the CHATS system (Aslam et al. 1992; Kronzucker et al. 1995).

At higher nitrate concentrations (>0.25 and as high as 50 mmol·L$^{-1}$ nitrate) a third transport system, the LATS becomes apparent. In barley roots, in contrast to the IHATS, the LATS is constitutive, and as stated above, displays a linear concentration-dependence (Siddiqi et al. 1990). By contrast, in *A. thaliana* the LATS appears to be inducible (Tsay et al. 1993). The linear LATS was at first interpreted as the signature of a passive (even diffusive) entry of nitrate. However, evaluations based upon measured electrical potentials and estimated cytoplasmic nitrate indicate that even at high external nitrate, this linear pattern of $NO_3^-$ uptake is thermodynamically active (Glass et al. 1992). In summary, physiological studies suggested three discrete types of transport system for nitrate, all thermodynamically active, some constitutive, some nitrate inducible.

### Attempts at biochemical characterizations

In the 1980s, several groups attempted to isolate putative nitrate transporters by making use of the fact that exposure of nitrate-deprived plants to nitrate increased nitrate transport so dramatically. SDS gels of solubilized membrane fractions isolated from roots before and after exposure to nitrate were scoured for the appearance of novel proteins (McClure et al. 1987), but this was to no avail. It was necessary to await the developments in molecular genetics and their application to plant systems before success would be realized.

## Molecular methods

### *CHL1* (*NRT1.1*)

The first gene encoding a plant nitrate transporter was isolated from *A. thaliana*. This achievement built upon the mutant work of Doddema and Telkamp (1979). Chlorate ($ClO_3^-$) is an analogue of $NO_3^-$, even having seen use as a herbicide because plants absorb $ClO_3^-$ and reduce it to $ClO_2^-$, with toxic results. Doddema and Telkamp (1979) characterized a mutant (*B1*) defective in LATS nitrate uptake, which was isolated by its capacity to survive chlorate treatment. Interestingly they also obtained nitrate reductase mutants by this method. Tsay et al. (1993) isolated a T-DNA insertional mutant of *A. thaliana* that like *B1* was able to survive $ClO_3^-$ treatment. This enabled them to isolate a nitrate-inducible *CHL1* gene (*AtNRT1.1*) and to demonstrate its capacity for nitrate uptake in the *Xenopus* oocyte system. Interestingly, when grown on $KNO_3$, as opposed to $NH_4NO_3$, Touraine and Glass (1997) failed to observe reduced $^{13}NO_3^-$ LATS fluxes in the *Atnrt1.1* mutant. Indeed, even when grown on $NH_4NO_3$, LATS influx, although reduced, was not eliminated suggesting that other genes might contribute to LATS. This anomaly was partly resolved when Huang et al. (1999) cloned a second *NRT1* gene (*AtNRT1.2*) involved in constitutive low-affinity transport. Subsequently, as many as 53 other members of the *NRT1* family have been recognized in

*A. thaliana*. The family contains members that are nitrate transporters, whereas others are dipeptide transporters (reviewed in Tsay et al. 2007). Of these, *AtNRT1.1* and *AtNRT1.2* are involved in nitrate uptake (Huang et al. 1999), *AtNRT1.4* participates in petiole nitrate storage (Chiu et al. 2004), *AtNRT1.5* is involved in xylem nitrate loading (Tsay et al. 2007), and *AtNRT1.6* plays a role in early embryo development (Almagro et al. 2008)

*AtNRT1.1* is subject to phosphorylation/dephosphorylation cycles at threonine residue 101, which is claimed to cause the transporter to cycle between a HATS and a LATS (Liu and Tsay 2003). Hence, the term dual-affinity nitrate transporter has been applied to the *AtNRT1.1* gene product and thus it has been proposed that *AtNRT1.*1 plays a major role in HATS in addition to its role in LATS (Wang et al. 1998; Liu et al. 1999). Recently, it has been proposed that, in addition to its function as a nitrate transporter, *AtNRT1.1* participates in the control of root proliferation in response to elevated soil nitrate (Remans et al. 2006*a*). This is based upon the finding that *AtNRT1.1* mutants display reduced lateral root elongation into such regions.

### *NRT2* genes

Chlorate survival was also used to generate a $NO_3^-$ uptake mutant from the fungus *Aspergillus nidulans* (Eidam) G. Winter. The mutant was named *crnA* and enabled Unkles et al. (1991) to isolate the first gene (initially named *CRNA*, and subsequently *NRTA*) encoding a eukaryotic HATS. Subsequently, a second gene encoding high-affinity $NO_3^-$ transport (*NRTB*) was isolated from *A. nidulans* (Unkles et al. 2001). Kinetic properties of these transporters are quite different and *NRTB* shows much higher $NO_2^-$ than $NO_3^-$ fluxes. These genes belong to a major facilitator superfamily (MFS), having 12 putative membrane-spanning domains (Forde 2000). Soon after the cloning of *NRTA*, mutants of *Chlamydomonas* revealed that two genes encoded $NO_3^-$ HATS in this species (Quesada et al. 1994). Initially, these were called *NAR3* and *NAR4*; subsequently, they were renamed *NRT2.1* and *NRT2.2*. Using degenerative primers (Trueman et al. 1996; Zhuo et al. 1999) and differential induction using nitrate and glutamine (Filleur and Daniel-Vedele 1999), corresponding members of the *NRT2* family were isolated from barley and from *A. thaliana*. *NRT2* homologues have now been isolated from many species, including *Nicotiana plumbaginofolia* Viv. (Quesada et al. 1997; Krapp et al. 1998), soybean (Amarasinghe et al. 1998), tomato (Ono et al. 2000), *Chlorella sorokiniana* (Koltermann et al. 2003), *Dunaliella* (He et al. 2004), rice (Miller et al. 2007; Cai et al. 2008), wheat (Zhao et al. 2004; Yin et al. 2007), and the moss *Physcomitrella patens* (Hedw.) Bruch & Schimp (Tsujimoto et al. 2007).

By use of degenerative primers, Zhuo et al. (1999) demonstrated the presence of a second *NRT2* (*NRT2.2*) homologue that was in tail-to-tail configuration with *NRT2.1*. Completion of the *A. thaliana* genome project subsequently revealed that in total, this species possessed as many as seven *NRT2* homologues. Expression patterns of these genes in both roots and shoots were evaluated by Orsel et al. (2002) and by Okamoto et al. (2003). Strong correlations were observed between patterns of high-affinity $NO_3^-$ influx and *NRT2.1* expression both in *A. thaliana* and in barley. Further strong support for the primacy of the role of *Atnrt2.1* in IHATS came with the demonstration that a T-DNA mutant disrupted in *AtNRT2.1* exhibited a 67% reduction of $^{15}NO_3^-$ (Filleur et al. 2001) and reduced growth when the external concentration of nitrate was low (Orsel et al. 2004). One problem with this particular mutant was that although it suffered a major disruption in both *NRT2.1* and *NRT2.2*, in ascribing the *IHATS* to *NRT2.1* the authors treated their findings as though the mutation was exclusively in *NRT2.1*. Nevertheless, further study with mutants specifically disrupted in *NRT2.1* or *NRT2.2* confirmed the primary importance of *NRT2.1*, but indicated that disruption of *NRT2.2* reduced $^{13}NO_3^-$ uptake by up to 19% and that expression levels of *NRT2.2* were increased three-fold when *NRT2.1* was disrupted (Li et al. 2007), presumably compensating for the disruption of *NRT2.1*. Furthermore, growth of the *nrt2.1/nrt2.2* mutant was consistently poorer than that of the *nrt2.1* mutant on low-nitrate agar, again suggesting a contributory role for *NRT2.2* in IHATS. Interestingly, following resupply of nitrate to nitrate-starved plants, *AtNRT2.2* (like *AtNRT2.1*) initially showed a rapid increase of transcript abundance, followed after about 3 h by a rapid decline (Okamoto et al. 2003). By contrast *AtNRT2.1* declined slowly over several days.

## Morphological considerations

A link between mineral nutrition (and even availability of water) and root morphology has been recognized for over a century (Miller 1938). Typically, root growth proliferates and root:shoot ratios increase when either water or various nutrients (including nitrate) are in short supply. Drew and Goss (1974) observed that roots also proliferated in regions rich in various nutrients when these were interspersed with zones without nutrients. Recently, it has been demonstrated that as well as functioning in nitrate transport, *AtNRT2.1* may serve to integrate lateral root initiation and lateral root growth (Little et al. 2005; Remans et al. 2006*b*). Clearly, the processes of nitrate uptake must be intimately integrated with the morphological responses to available sources of nitrate. It is perhaps not surprising then to find that the genes encoding both *Atnrt2.1* and *Atnrt1.1* themselves may be also be participants in these nitrate signalling pathways.

### Regulation of *NRT2* expression

The correlations between $^{13}NO_3^-$ influx and *NRT2* abundance in roots of barley or *A. thaliana* are evident during induction and subsequent down regulation or in plants maintained at different steady-state levels of nitrate supply (Zhuo et al. 1999). Attempts to identify the source of this feedback inhibition long preceded the identification of the genes responsible for IHATS. Both accumulated nitrate and end-products of nitrate assimilation (particularly glutamine) were suggested to be responsible (reviewed in Glass and Siddiqi 1995). Following the cloning of genes encoding IHATS, researchers supplied various nitrogen forms such a $NO_3^-$, $NH_4^+$, and various amino acids, then measured $NO_3^-$ influx and *NRT2* transcript abundance with a view to identifying which N pool(s) regulated nitrate uptake. Unfortunately, the rapid assimilation of $NO_3^-$ and $NH_4^+$, and the rapid interconversion of amino acids make it virtually im-

possible to identify the N pool or pools responsible for down regulation using this methodology. Zhuo et al. (1999) and Vidmar et al. (2000), refined this approach by adding various inhibitors of nitrogen interconversion together with these N forms. Surprisingly, when methionine sulfoximine (an inhibitor of $NH_4^+$ assimilation via glutamine synthetase) was employed, the reduction of transcript abundance normally observed following application of $NH_4^+$ was much reduced. This suggested that the effects of $NH_4^+$ are not mediated by $NH_4^+$ per se. In the presence of azaserine, an inhibitor of the conversion of glutamine to glutamate, nitrate influx and *NRT2* transcript were virtually abolished, strongly suggesting that glutamine is the responsible regulator. In a recent paper by Tsujimoto et al. (2007), these results were confirmed using the moss *Physcomitrella patens*. The authors concluded that glutamine is the signal for repression of both *PpNRT2* and *PpNar2* genes (see below). Likewise, in a study of high-affinity $NH_4^+$ uptake in *A. thaliana* it was demonstrated that despite an almost 30-fold increase of root $NH_4^+$ concentration in the presence of methionine sulfoximine, transcript abundance of the high-affinity $NH_4^+$ transporter gene (*AMT1.1*) and $^{13}NH_4^+$ influx remained almost at control levels (Rawat et al. 1999). A role for the *Atnrt1.1* transporter in mediating the normal repression of *AtNRT2.1* by high N has been proposed by Munos et al. (2004) on the basis of the elevated HATS activity exhibited by *chl1–5* (*AtNRT1.1*) mutants subjected to elevated levels of N.

Because of the requirement for carbon skeletons to assimilate incoming nitrate to the level of amino acids, it appears that carbon availability also influences *NRT2* transcript abundance and nitrate influx. Thus, at night both *NRT2.1* transcript abundance and nitrate influx decline in *A. thaliana* and tobacco and can be partially restored by exogenous application of sucrose (Lejay et al.1999; Matt et al. 2001). Indeed this phenomenon is common to $NH_4^+$ uptake by *Atamt1.3* in *A. thaliana*, *Leamt1.2* in tomato, and *Osamt1.3* in rice (Gazzarrini et al. 1999; von Wiren et al. 2000; Kumar et al. 2003).

### Interactions between *AtNRT2* and *AtNAR2* (*AtNRT3.1*)

In *Chlamydomonas NRT2.1* or *NRT2.2*, genes encoding the HATS were only functional when a considerably smaller gene, *CrNAR2* (encoding a membrane protein with a molecular mass of roughly 25 kDa), was simultaneously expressed. Although *NRT2.1*, *NRT2.2*, and *NRTA* share considerable sequence identity, they are unrelated to *NAR2*. As yet, the function of this membrane protein is unknown. Unlike nrt2 proteins that are thought to possess 12 transmembrane domains (TMD), *NAR2* appears to have a single TMD. Using the *Xenopus* oocyte system, it was demonstrated that nitrate transport was realized only when the *Chlamydomonas NRT2.1* was co-injected with *CrNAR2* cDNA, providing supporting evidence for the interaction between these two proteins (Zhou et al. 2000*a*). Further in planta support for this concept came from an *A. thaliana* T-DNA mutant disrupted in *AtNAR2.1* (Okamoto et al. 2006). This gene was renamed *AtNRT3.1* because the *NAR2* name had already been selected for another gene in this species. IHATS in this *A. thaliana* *AtNRT3.1* mutant was virtually eliminated as was the CHATS, providing circumstantial evidence that the CHATS protein also requires coexpression of *AtNRT3.1*. By contrast, LATS activity in the mutant was intact. In these mutants, *AtNRT2.1* transcript abundance, although reduced, was still significant; thus *AtNRT3.1* does not act through transcriptional regulation of *AtNRT2.1*. The reduction in *AtNRT2.1* transcript was assumed to be the result of reduced nitrate uptake in these mutants. Further evidence for the interaction between *NRT2.1* and *NRT3.1* was provided by a positive yeast split-ubiquitin reaction using histidine auxotrophy and β-galactosidase assays (Glass et al. 2004). This finding was confirmed by Orsel et al. (2006). The identification of *NRT2* and *NAR2* homologues in rice (Araki and Hasegawa 2006; Miller et al. 2007; Cai et al. 2008), wheat (Cai et al. 2007), barley (Tong et al. 2005), and *Physcomitrella patens* (Tsujimoto et al. 2007) suggests that the two-protein system for $NO_3^-$ uptake is probably universal among plants, as well as in *Chlamydomonas*. However, in *A. nidulans* there appears to be no *nar2* requirement because when expressed in the *Xenopus* system the *nrtA* (formerly *crnA*) transporter resulted in the flux of nitrate, without the necessity of co-expressing *nar2* (Zhou et al. 2000*b*). Since this work was completed, we have demonstrated a positive yeast split-ubiquitin reaction using histidine auxotrophy and β-galactosidase assays using *AtNRT2.2* and *AtNRT3.1*, and the requirement for both *Atnrt2.2* and *Atnrt3.1* cDNAs for $^{15}NO_3^-$ accumulation in *Xenopus* oocytes (W. Li and A.D.M. Glass, unpublished data). Thus, *Atnrt2.2* also appears to interact with *Atnrt3.1* for normal function.

Nazoa et al. (2003) demonstrated that *AtNRT2.1* transcript is localized in epidermal and cortical cells, specifically in tissues back from the root tip. Recently, Wirth et al. (2007) used *AtNRT2.1*-GFP transgenic lines to demonstrate that *Atnrt2.1* is mainly localized in the plasma membrane of epidermal and cortical cells. They also identified, via the use of immunological methods, a number of higher molecular weight forms of *Atnrt2.1*, but failed to detect any putative *Atnrt2.1*/*nrt3.1* complex that might constitute the anticipated functional nitrate transporter. Interestingly, the most abundant form of *Atnrt2.1*, a 45 kDa polypeptide appeared to be lost in *Atnrt3.1* mutants, suggesting that *Atnrt3.1* is required for the *Atnrt2.1* to be expressed and addressed to the plasma membrane. Finally, the authors observed that conditions well known to reduce *Atnrt2.1* transcript abundance and nitrate influx (e.g., light, sucrose, nitrogen treatments) failed to affect *Atnrt2.1* at the protein level. The authors suggested that these observations supported previous claims (Fraisier et al. 2000; Vidmar et al. 2000) that *Atnrt2.1* is regulated post-transcriptionally, as well as transcriptionally. In a paper devoted to the fungus *A. nidulans* (Wang et al. 2007), it was demonstrated that in a nitrate reductase mutant (*NiaD171*), transcript abundance of the *NRTA* gene encoding the HATS and *nrtA* protein was at levels more than two-fold and three-fold higher, respectively, than in wild-type (WT) strains, yet $^{13}NO_3^-$ influx was approximately 5% of WT. The authors concluded that such a reduction of influx, given elevated protein and mRNA, could only be explained by post-translational regulation.

## Other *NRT2* genes

Chopin et al. (2007) demonstrated that *AtNRT2.7* is highly expressed in dry seeds and postulated a role for nitrate up-

take in this reproductive tissue. Mutant lines accumulated less nitrate than WT and had delayed germination. When *AtNRT2.7* was expressed in a *AtNRT2.1*/*AtNRT2.2* double mutant and in the *Xenopus* oocyte system, nitrate uptake was increased above control levels, suggesting that *Atnrt2.7* is capable of nitrate transport. The use of a GFP-fusion protein indicated that *Atnrt2.7* was localized to the tonoplast of mature cells. T-DNA mutants disrupted in *AtNRT2.6* have been investigated for both $^{13}NO_3^-$ and $^{13}NO_2^-$ influx into roots and translocation of $^{13}N$ to shoots (R. Nandanavanam and A.D.M. Glass, unpublished data). Neither flux was reduced, but there was a small but significant reduction (~20%) in root and shoot biomass, which was normalized by transforming *AtNRT2.6* into the mutant.

In conclusion, contemporary developments in molecular biology have resulted in the identification of genes encoding both the IHATS and the LATS in *A. thaliana,* as well as in other species. The third of the physiologically defined systems (CHATS) was identified in the form of a mutant (Wang and Crawford 1996) that is defective in CHATS, but the gene encoding this function remains to be identified. Other major topics still to be clarified include the roles of the remaining *NRT2* genes, details of their regulation and of the interactions between *nrt2.1*/*nrt2.2* and *nrt3.1*.

## References


Almagro, A., Lin, S.-H., and Tsay, Y.-F. 2008. Characterization of the *Arabidopsis* NRT1.6 reveals a role of nitrate in early embryo development. Plant Cell, **20**: 3289–3299. PMID:19050168.

Amarasinghe, B.H.R.R., de Bruxelles, G.L., Braddon, M., Onyeocha, I., Ford, B.G., and Udvardi, M.K. 1998. Regulation of *GmNRT2* expression and nitrate transport activity in roots of soybean (*Glycine max*). Planta, **206**: 44–52. doi:10.1007/s004250050372.

Araki, R., and Hasegawa, H. 2006. Expression of rice (*Oryza sativa* L.) genes involved in high-affinity nitrate transport during the period of nitrate induction. Breed. Sci. **56**: 295–302. doi:10.1270/jsbbs.56.295.

Aslam, M., Travis, R.L., and Huffaker, R.C. 1992. Comparative kinetics and reciprocal inhibition of nitrate and nitrite uptake in roots of uninduced and induced barley (*Hordeum vulgare* L.) seedlings. Plant Physiol. **99**: 1124–1133. doi:10.1104/pp.99.3.1124. PMID:11537883.

Behl, R., Tischner, R., and Raschke, K. 1988. Induction of a high-capacity nitrate uptake mechanism in barley roots prompted by nitrate uptake through a constitutive low-capacity mechanism. Planta, **176**: 235–240. doi:10.1007/BF00392450.

Cai, C., Zhao, X.Q., Zhu, Y.G., Li, B., Tong, Y.P., and Li, Z.S. 2007. Regulation of the high-affinity nitrate transport system in wheat roots by exogenous abscisic acid and glutamine. J. Integr. Plant Biol. **49**: 1719–1725. doi:10.1111/j.1744-7909.2007.00485.x.

Cai, C., Wang, J.Y., Zhu, Y.-G., Shen, Q.-R., Li, B., Tong, Y.P., and Li, Z.-S. 2008. Gene structure and expression of the high-affinity nitrate transport system in rice roots. J. Integr. Plant Biol. **50**: 443–451. doi:10.1111/j.1744-7909.2008.00642.x. PMID:18713378.

Chiu, C.C., Lin, C.S., Hsia, A.P., Su, R.C., Lin, H.L., and Tsay, Y.-F. 2004. Mutation of a nitrate transporter, AtNRT1.4, results in a reduced petiole nitrate content and altered leaf development. Plant Cell Physiol. **45**: 1139–1148. doi:10.1093/pcp/pch143. PMID:15509836.

Chopin, F., Orsel, M., Dorbe, M.F., Chardon, F., Truong, H.-N., Miller, A.J., Krapp, A., and Daniel-Vedele, F. 2007. The *Arabidopsis* ATNRT2.7 nitrate transporter controls nitrate content in seeds. Plant Cell, **19**: 1590–1602. doi:10.1105/tpc.107.050542. PMID:17540716.

Cram, W.J. 1976. Negative feedback regulation of transport in cells. The maintenance of turgor, volume and nutrient supply. *In* Encyclopedia of plant physiology. New Series IA. *Edited by* U. Luttge and M.G. Pitman. Springer-Verlag, New York, N.Y. pp. 284–316.

Doddema, H., and Telkamp, G.P. 1979. Uptake of nitrate by mutants of *Arabidopsis thaliana* disturbed in uptake or reduction of nitrate. II. Kinetics. Physiol. Plant. **45**: 332–338. doi:10.1111/j.1399-3054.1979.tb02593.x.

Drew, M.C., and Goss, M.J. 1974. Environmental stress and the growth of the barley root systems: the effect of nutrient ion concentration and mechanical impedance. *In* Mechanisms of regulation of plant growth. *Edited by* R.L. Bieleski, A.R. Ferguson, and M.M. Creswell. R. Soc. N.Z. Bull. 12.

Epstein, E. 1972. Mineral nutrition of plants: principles and perspectives. Wiley, New York, N.Y.

Epstein, E. 1976. Kinetics of ion transport and the carrier concept. *In* Encyclopedia of plant physiology. New Series 2B. *Edited by* U. Luttge and M.G. Pitman. Springer-Verlag, New York, N.Y. pp. 70–94.

Filleur, S., and Daniel-Vedele, F. 1999. Expression analysis of a high-affinity nitrate transporter isolated from *Arabidopsis thaliana* by differential display. Planta, **207**: 461–469. doi:10.1007/s004250050505. PMID:9951738.

Filleur, S., Dorbe, M.F., Cerezo, M., Orsel, M., Granier, F., Gojon, A., and Daniel-Vedele, F. 2001. An *Arabidopsis* T-DNA mutant affected in *Nrt2* genes is impaired in nitrate uptake. FEBS Lett. **489**: 220–224. doi:10.1016/S0014-5793(01)02096-8. PMID:11165253.

Forde, B.G. 2000. Nitrate transporters in plants: structure, function and regulation. Biochim. Biophys. Acta, **1465**: 219–236. doi:10.1016/S0005-2736(00)00140-1. PMID:10748256.

Fraisier, V., Gojon, A., Tillard, P., and Daniel-Vedele, F. 2000. Constitutive expression of a putative high-affinity nitrate transporter in *Nicotiana plumbaginifolia*: evidence for post-transcriptional regulation by a reduced nitrogen source. Plant J. **23**: 489–496. doi:10.1046/j.1365-313x.2000.00813.x. PMID:10972875.

Galván, A., and Fernández, E. 2001. Eukaryotic nitrate and nitrite transporters. Cell. Mol. Life Sci. **58**: 225–233. doi:10.1007/PL00000850. PMID:11289304.

Galván, A., Quesada, A., and Fernández, E. 1996. Nitrate and nitrite are transported by different specific transport systems and by a bispecific transporter in *Chlamydomonas reinhardtii*. J. Biol. Chem. **271**: 2088–2092. doi:10.1074/jbc.271.4.2088. PMID:8567664.

Gazzarrini, S., Lejay, L., Gojon, A., Ninnemann, O., Frommer, W.B., and von Wirén, N. 1999. Three functional transporters for constitutive, diurnally regulated, and starvation-induced uptake of ammonium into *Arabidopsis* roots. Plant Cell, **11**: 937–948. doi:10.1105/tpc.11.5.937. PMID:10330477.

Gessler, A., Schneider, S., Von Sengbusch, D., Weber, P., Hanemann, U., Huber, C., Rothe, A., Kreutzer, K., and Rennenberg, H. 1998. Field and laboratory experiments on net uptake of nitrate and ammonium by the roots of spruce (*Picea abies*) and beech (*Fagus sylvatica*) trees. New Phytol. **138**: 275–285. doi:10.1046/j.1469-8137.1998.00107.x.

Glass, A.D.M., Siddiqi, Y., Kumar, A., Li, W., Wang, Y., Okamoto, M., Unkles, S., and Kinghorn, J.R. 2004. Nitrate uptake by plant roots: making the connection between physiology and

molecular biology. *In* Proceedings of the 7th International Symposium on Inorganic Nitrogen Assimilation in Plants: from the Genome to the Agro-Ecosystem. *Edited by* I. Stulen. 23–27 June 2004. Wageningen, the Netherlands. Groningen University Press, Groningen, Netherlands.

Glass, A.D.M., and Siddiqi, M.Y. 1995. Nitrogen absorption by plant roots *In* Nitrogen nutrition in higher plants. *Edited by* H.S. Srivastava and R.P. Singh. Associated Publishing Co., New Delhi, India. pp. 21–56

Glass, A.D.M., Shaff, J.E., and Kochian, L.V. 1992. Studies of nitrate uptake in barley. IV. Electrophysiology. Plant Physiol. **99**: 456–463. doi:10.1104/pp.99.2.456. PMID:16668907.

Guy, M., and Heimer, Y.M. 1993. On the inducibility of nitrate transport by tobacco cells. Physiol. Plant. **89**: 596–601. doi:10.1111/j.1399-3054.1993.tb05220.x.

He, Q.H., Qiao, D.R., Zhang, Q.L., Li, Y., Xu, H., Wei, L., Gu, Y., and Cao, Y. 2004. Cloning and expression study of a putative high-affinity nitrate transporter gene from *Dunaliella salina*. J. Appl. Phycol. **16**: 395–400. doi:10.1023/B:JAPH.0000047950.76549.ce.

Higinbotham, N., Etherton, B., and Foster, R.J. 1967. Mineral ion content and cell transmembrane electropotentials of pea and oat seedling tissue. Plant Physiol. **42**: 37–46. doi:10.1104/pp.42.1.37. PMID:16656483.

Hoagland, D.R., and Broyer, T.C. 1936. General nature of the process of salt accumulation by roots with description of experimenetal methods. Plant Physiol. **11**: 471–507. doi:10.1104/pp.11.3.471. PMID:16653362.

Hoagland, D.R., Hibbard, P.L., and Davies, A.R. 1926. The influence of light, temperature and other conditions on the ability of *Nitella* cells to concentrate halogens in the cell sap. J. Gen. Physiol. **10**: 121–146. doi:10.1085/jgp.10.1.121.

Huang, N.-C., Liu, K.-H., Lo, H.-J., and Tsay, Y.-F. 1999. Cloning and functional characterization of an *Arabidopsis* nitrate transporter gene that encodes a constitutive component of low-affinity uptake. Plant Cell, **11**: 1381–1392. doi:10.1105/tpc.11.8.1381. PMID:10449574.

Jackson, W.A., Volk, R.J., and Tucker, T.C. 1972. Apparent induction of nitrate uptake in nitrate-depleted plants. Agron. J. **64**: 518–521.

Kielland, K. 1994. Amino acid absorption by artic plants: implications for plant nutrition and nitrogen cycling. Ecology, **75**: 2373–2383. doi:10.2307/1940891.

King, B.J., Siddiqi, M.Y., Ruth, T.J., Warner, R.L., and Glass, A.D.M. 1993. Feedback regulation of nitrate influx in barley roots by nitrate, nitrate and ammonium. Plant Physiol. **102**: 1279–1286. PMID:12231904.

Koltermann, M., Moroni, A., Gazzarini, S., Nowara, D., and Tischner, R. 2003. Cloning, functional expression and expression studies of the nitrate transporter gene from *Chlorella sorokiniana* (strain 211-8k). Plant Mol. Biol. **52**: 855–864. doi:10.1023/A:1025024821832. PMID:13677472.

Krapp, A., Fraisier, V., Scheible, W.R., Quesada, A., Gojon, A., Stitt, M., Caboche, M., and Daniel-Vedele, F. 1998. Expression studies of Nrt2:1Np, a putative high-affinity nitrate transporter: evidence for its role in nitrate uptake. Plant J. **14**: 723–731. doi:10.1046/j.1365-313x.1998.00181.x.

Kronzucker, H.J., Siddiqi, M.Y., and Glass, A.D.M. 1995. Compartmentation and flux characteristics of nitrate in spruce. Planta, **196**: 674–682. doi:10.1007/BF01106760.

Kronzucker, H.J., Siddiqi, M.Y., and Glass, A.D.M. 1997. Root discrimination against soil nitrate and the ecology of forest succession. Nature (London), **385**: 59–61. doi:10.1038/385059a0.

Kumar, A., Silim, S.N., Okamoto, M., Siddiqi, M.Y., and Glass, A.D.M. 2003. Differential expression of three members of the *AMT1* gene family encoding putative high-affinity $NH_4^+$ transporters in roots of *Oryza sativa* subspecies indica. Plant Cell Environ. **26**: 907–914. doi:10.1046/j.1365-3040.2003.01023.x. PMID:12803618.

Lee, R.B. 1979. The effect of nitrite on root growth of barley and maize. New Phytol. **83**: 615–622. doi:10.1111/j.1469-8137.1979.tb02293.x.

Lejay, L., Tillard, P., Lepetit, M., Olive, F.D., Filleur, S., Daniel-Vedele, F., and Gojon, A. 1999. Molecular and functional regulation of two $NO_3^-$ uptake systems by N- and C-status of *Arabidopsis* plants. Plant J. **18**: 509–519. doi:10.1046/j.1365-313X.1999.00480.x. PMID:10417701.

Li, W., Wang, Y., Okamoto, M., Crawford, N.M., Siddiqi, M.Y., and Glass, A.D.M. 2007. Dissection of the *AtNRT2.1*:*AtNRT2.2* inducible high-affinity nitrate transporter gene cluster. Plant Physiol. **143**: 425–433. doi:10.1104/pp.106.091223. PMID:17085507.

Little, D.Y., Rao, H., Oliva, S., Daniel-Vedele, F., Krapp, A., and Malamy, J.E. 2005. The putative high-affinity nitrate transporterNRT2.1 represses lateral root initiation in response to nutritional cues. Proc. Natl. Acad. Sci. U.S.A. **102**: 13693–13698. doi:10.1073/pnas.0504219102. PMID:16157886.

Liu, K.-H., and Tsay, Y.-F. 2003. Switching between the two action modes of the dual-affinity nitrate transporter CHL1 by phosphorylation. EMBO J. **22**: 1005–1013. doi:10.1093/emboj/cdg118. PMID:12606566.

Liu, K.-H., Huang, C.-Y., and Tsay, Y.-F. 1999. *CHL1* is a dual-affinity nitrate transporter of *Arabidopsis* involved in multiple phases of nitrate uptake. Plant Cell, **11**: 865–874. doi:10.1105/tpc.11.5.865. PMID:10330471.

Mariscal, V., Rexach, J., Fernandez, E., and Galvan, A. 2004. The plastidic nitrite transporter NAR1;1 improves nitrate use efficiency for growth in *Chlamydomonas*. Plant Cell Environ. **27**: 1321–1328. doi:10.1111/j.1365-3040.2004.01239.x.

Matt, P., Geiger, M., Walch-Liu, P., Engels, C., Krapp, A., and Stitt, M. 2001. The immediate cause of the diurnal changes of nitrogen metabolism in leaves of nitrate-replete tobacco: a major imbalance between the rate of nitrate reduction and the rates of nitrate uptake and ammonium metabolism during the first part of the light period. Plant Cell Environ. **24**: 177–190. doi:10.1111/j.1365-3040.2001.00676.x.

McClure, P.R., Ohmolt, T.E., Pace, G.M., and Bouthyette, P.-Y. 1987. Nitrate-induced changes in protein synthesis and translocation of RNA in maize roots. Plant Physiol. **84**: 52–57. doi:10.1104/pp.84.1.52. PMID:16665404.

Miller, E.C. 1938. Plant physiology: with reference to the green plant. McGraw-Hill, New York, N.Y.

Miller, A., Smith, S., Fan, X., and Shen, Q. 2007. Expression and functional analysis of rice NRT2 nitrate transporters. Comp. Biochem. Physiol. **146**: S241.

Minotti, P.L., Williams, D.C., and Jackson, W.A. 1969. Nitrate uptake by wheat as influenced by ammonium and other cations. Crop Sci. **9**: 9–14.

Munos, S., Cazettes, C., Fizames, C., Gaymard, F., Tillard, P., Lepetit, M., Lejay, L., and Gojon, A. 2004. Transcript profiling in the chl1–5 mutant of *Arabidopsis* reveals a role of the nitrate transporter NRT1.1 in the regulation of another nitrate transporter, NRT2.1. Plant Cell, **16**: 2433–2447. doi:10.1105/tpc.104.024380. PMID:15319483.

Nazoa, P., Vidmar, J.J., Tranbarger, T.J., Mouline, K., Damiani, I., Tillard, P., Zhuo, D., Glass, A.D.M., and Touraine, B. 2003. Regulation of the nitrate transporter gene *AtNRT2.1* in *Arabidopsis thaliana*: responses to nitrate, amino acids and develop-

mental stage. Plant Mol. Biol. **52**: 689–703. doi:10.1023/A:1024899808018. PMID:12956537.

Needoba, J.A., Sigman, D.M., and Harrison, P.J. 2004. The mechanism of isotope fractionation during algal nitrate assimilation as illuminated by the $^{15}N/^{14}N$ of intracellular nitrate. J. Phycol. **40**: 517–522.

Okamoto, M., Vidmar, J.J., and Glass, A.D.M. 2003. Regulation of *NRT1* and *NRT2* gene families of *Arabidopsis thaliana*: responses to nitrate provision. Plant Cell Physiol. **44**: 304–317. doi:10.1093/pcp/pcg036. PMID:12668777.

Okamoto, M., Kumar, A., Li, W., Wang, Y., Siddiqi, M.Y., Crawford, N.M., and Glass, A.D.M. 2006. High-affinity nitrate transport in roots of *Arabidopsis* depends on expression of the *NAR2*-like gene *AtNRT3.1*. Plant Physiol. **140**: 1036–1046. doi:10.1104/pp.105.074385. PMID:16415212.

Ono, F., Frommer, W.B., and von Wiren, N. 2000. Coordinated diurnal regulation of low- and high-affinity nitrate transporters in tomato. Plant Biol. **2**: 17–23. doi:10.1055/s-2000-297.

Orsel, M., Krapp, A., and Daniel-Vedele, F. 2002. Analysis of the NRT2 nitrate transporter family in *Arabidopsis*. Structure and gene expression. Plant Physiol. **129**: 886–896. doi:10.1104/pp.005280. PMID:12068127.

Orsel, M., Eulenburg, K., Krapp, A., and Daniel-Vedele, F. 2004. Disruption of the nitrate transporter genes *AtNRT2.1* and *AtNRT2.2* restricts growth at low external nitrate concentration. Planta, **219**: 714–721. doi:10.1007/s00425-004-1266-x. PMID:15107992.

Orsel, M., Chopin, F., Leleu, O., Smith, S.J., Krapp, A., Daniel-Vedele, F., and Miller, A.J. 2006. Characterization of a two-component high-affinity nitrate uptake system in *Arabidopsis*. Physiology and protein-protein interaction. Plant Physiol. **142**: 1304–1317. doi:10.1104/pp.106.085209. PMID:17012411.

Overton, E. 1895. Über die osmotischen Eigenschaften der lebenden Pflanzen- und Tierzelle. Vierteljahrsschr. Naturforsch. Ges. Zuer. **40**: 159–201.

Pace, G.M., and McClure, P.R. 1986. Comparison of nitrate uptake kinetic parameters across maize inbred lines. J. Plant Nutr. **9**: 1095–1111. doi:10.1080/01904168609363512.

Poole, R.J. 1978. Energy coupling for membrane transport. Annu. Rev. Plant Physiol. **29**: 437–460. doi:10.1146/annurev.pp.29.060178.002253.

Quesada, A., Galván, A., and Fernandez, E. 1994. Identification of nitrate transporter genes in *Chlamydomonas reinhardtii*. Plant J. **5**: 407–419. doi:10.1111/j.1365-313X.1994.00407.x. PMID:8180624.

Quesada, A., Krapp, A., Trueman, L.J., Daniel-Vedele, F., Fernandez, E., Forde, B.G., and Caboche, M. 1997. PCR-identification of a *Nicotiana plumbaginifolia* cDNA homologous to the high-affinity nitrate transporters of the crnA family. Plant Mol. Biol. **34**: 265–274. doi:10.1023/A:1005872816881. PMID:9207842.

Rawat, S.R., Silim, S.L., Kronzucker, H.J., Siddiqi, M.Y., and Glass, A.D.M. 1999. *AtAMT1* gene expression and $NH_4^+$ uptake in roots of *Arabidopsis thaliana*: evidence for regulation by root glutamine levels. Plant J. **19**: 143–152. doi:10.1046/j.1365-313X.1999.00505.x. PMID:10476061.

Remans, T., Nacry, P., Pervent, M., Filleur, S., Diatloff, E., Mounier, E., Tillard, P., Forde, B.G., and Gojon, A. 2006*a*. The *Arabidopsis* NRT1.1 transporter participates in the signalling pathway triggering root colonization of nitrate-rich patches. Proc. Natl. Acad. Sci. U.S.A. **103**: 19206–19211. doi:10.1073/pnas.0605275103. PMID:17148611.

Remans, T., Nacry, P., Pervent, M., Girin, T., Tillard, P., Lepetit, M., and Gojon, A. 2006*b*. A central role for the nitrate transporter NRT2.1 in the integrated morphological and physiological responses of the root system to nitrogen limitation in *Arabidopsis*. Plant Physiol. **140**: 909–921. doi:10.1104/pp.105.075721. PMID:16415211.

Siddiqi, M.Y., Glass, A.D.M., Ruth, T.J., and Rufty, T.W. 1990. Studies of the uptake of nitrate in barley. I: Kinetics of $^{13}NO_3^-$ influx. Plant Physiol. **93**: 1426–1432. doi:10.1104/pp.93.4.1426. PMID:16667635.

Stark, J.M., and Hart, S.C. 1997. High rates of nitrification and nitrate turnover in undisturbed coniferous forests. Nature (London), **385**: 61–64. doi:10.1038/385061a0.

Stiles, W. 1924. Permeability. New Phytol. **13**: 1–296. [Reprint.]

Tong, Y., Zhou, J.-J., Li, Z., and Miller, A.J. 2005. A two-component high-affinity nitrate uptake system in barley. Plant. J. **41**(3): 442–450. doi:10.1111/j.1365-313X.2004.02310.x.

Touraine, B., and Glass, A.D.M. 1997. Nitrate and chlorate fluxes in the *chl1-5* mutant of *Arabidopsis thaliana*. Does the *CHL1-5* gene encode a low-affinity nitrate transporter? Plant Physiol. **114**: 137–144. doi:10.1104/pp.114.1.137. PMID:9159946.

Trueman, L.J., Richardson, A., and Forde, B.G. 1996. Molecular cloning of higher plant homologues of the high-affinity nitrate transporters of *Chlamydomonas reinhardtii* and *Aspergillus nidulans*. Gene, **175**: 223–231. doi:10.1016/0378-1119(96)00154-0. PMID:8917103.

Tsay, Y.F., Schroeder, J.I., Feldmann, K.A., and Crawford, N.M. 1993. A herbicide sensitivity gene *CHL1* of *Arabidopsis* encodes a nitrate-inducible nitrate transporter. Cell, **72**: 705–713. doi:10.1016/0092-8674(93)90399-B. PMID:8453665.

Tsay, Y.-F., Chiu, C.-C., Tsai, C.-B., Ho, C.-H., and Hsu, P.-K. 2007. Nitrate transporters and peptide transporters. FEBS Lett. **581**: 2290–2300. doi:10.1016/j.febslet.2007.04.047. PMID:17481610.

Tsujimoto, R., Yamazaki, H., Maeda, S.-I., and Omata, T. 2007. Distinct roles of nitrate and nitrite in regulation of expression of the nitrate transport genes in the moss *Physcomitrella patens*. Plant Cell Physiol. **48**: 484–497. doi:10.1093/pcp/pcm019. PMID:17289796.

Unkles, S.E., Hawker, K.L., Grieve, C., Campbell, E.I., Montague, P., and Kinghorn, J.R. 1991. *crna* encodes a nitrate transporter in *Aspergillus nidulans*. Proc. Natl. Acad. Sci. U.S.A. **88**: 204–208. doi:10.1073/pnas.88.1.204. PMID:1986367.

Unkles, S.E., Zhuo, D., Siddiqi, M.Y., Kinghorn, J.R., and Glass, A.D.M. 2001. Apparent genetic redundancy facilitates ecological plasticity for nitrate transport. EMBO J. **20**: 6246–6255. doi:10.1093/emboj/20.22.6246.

van den Honert, T.H. 1937. Over eigenschappen van plantenwortels welke een rol spelen bij de opname van voedingngszanten. Natuurwet. Tijdschr. Ned. Indie, **97**: 150–162.

van den Honert, T.H., and Hooymans, J.J.M. 1955. On the absorption of nitrate by maize in water culture. Acta Bot. Neerl. **4**: 376–384.

Vidmar, J.J., Zhuo, D., Siddiqi, M.Y., Schjoerring, J.K., Touraine, B., and Glass, A.D.M. 2000. Regulation of *HvNRT2* expression and high-affinity nitrate influx in roots of *Hordeum vulgare* by ammonium and amino acids. Plant Physiol. **123**: 307–318. doi:10.1104/pp.123.1.307. PMID:10806247.

von Wiren, N., Lauter, F.R., Ninnemann, O., Gillissen, B., Walch-Liu, P., Engels, C., Jost, W., and Frommer, W.B. 2000. Differential regulation of three ammonium transporter genes by nitrogen in roots hairs and by light in leaves of tomato. Plant J. **21**: 167–175. doi:10.1046/j.1365-313x.2000.00665.x. PMID:10743657.

Wang, R., and Crawford, N.M. 1996. Genetic identification of a gene involved in constitutive, high-affinity nitrate transport in higher plants. Proc. Natl. Acad. Sci. U.S.A. **93**: 9297–9301. doi:10.1073/pnas.93.17.9297. PMID:8799195.

Wang, R., Liu, D., and Crawford, N.M. 1998. The *Arabidopsis* CHL1 protein plays a major role in nitrate uptake. Proc. Natl. Acad. Sci. U.S.A. **95**: 15134–15139. doi:10.1073/pnas.95.25.15134. PMID:9844028.

Wang, Y., Li, W., Siddiqi, Y., Kinghorn, J.R., Unkles, S.E., and Glass, A.D.M. 2007. Evidence for post-translational regulation of NRTA, the *Aspergillus nidulans* high-affinity nitrate transporter. New Phytol. **175**: 699–706. doi:10.1111/j.1469-8137.2007.02135.x. PMID:17688585.

Wirth, J., Chopin, F., Santoni, V., Viennois, G., Tillard, P., Krapp, A., Lejay, L., Daniel-Vedele, F., and Gojon, A. 2007. Regulation of root nitrate uptake at the NRT2.1 protein level in *Arabidopsis thaliana*. J. Biol. Chem. **282**: 23541–23552. doi:10.1074/jbc.M700901200. PMID:17573350.

Wyn Jones, R.G., Brady, C.J., and Speirs, J. 1979. Ionic and osmotic relations in plant cells. *In* Recent advances in the biochemistry of cereals. *Edited by* D.L. Laidman and R.G. Wyn Jones. Academic Press, New York, N.Y. pp. 63–103.

Yin, L.P., Li, P., Wen, B., Taylor, D., and Berry, J.O. 2007. Characterization and expression of a high-affinity nitrate system transporter gene (*TaNRT2.1*) from wheat roots, and its evolutionary relationship to other NTR2 genes. Plant Sci. **172**: 621–631. doi:10.1016/j.plantsci.2006.11.014.

Zhao, X.Q., Li, Y.J., Liu, J.Z., Li, B., Liu, Q.Y., Tong, Y.P., Li, J.Y., and Li, Y.S. 2004. Isolation and expression analysis of a high-affinity nitrate transporter *TaNRT2.3* from roots of wheat. Acta Bot. Sin. **46**: 347–354.

Zhou, J.J., Fernández, E., Galván, A., and Miller, A.J. 2000*a*. A high-affinity nitrate transport system from *Chlamydomonas* requires two gene products. FEBS Lett. **466**: 225–227. doi:10.1016/S0014-5793(00)01085-1. PMID:10682832.

Zhou, J.J., Trueman, L.J., Boorer, K.G., Theodoulou, F.L., Forde, B.G., and Miller, A.J. 2000*b*. A high-affinity fungal nitrate carrier with two transport mechanisms. J. Biol. Chem. **275**: 39894–39899. doi:10.1074/jbc.M004610200. PMID:10984478.

Zhuo, D., Okamoto, M., Vidmar, J.J., and Glass, A.D.M. 1999. Regulation of putative high-affinity nitrate transporter (*Nrt2:1At*) in roots of *Arabidopsis thaliana*. Plant J. **17**: 563–568. doi:10.1046/j.1365-313X.1999.00396.x. PMID:10205909.

MINIREVIEW / MINISYNTHÈSE

# Biological roles of cyclic-nucleotide-gated ion channels in plants: What we know and don't know about this 20 member ion channel family[1]

**Kimberley Chin, Wolfgang Moeder, and Keiko Yoshioka**

**Abstract:** Cyclic-nucleotide-gated ion channels (CNGCs) are nonselective cation channels that were first identified in vertebrate retinal photoreceptors and olfactory sensory neurons. The role of CNGCs in animals is well documented, and they are believed to localize to the plasma membrane as heterotetramers. So far, only six CNGC genes (CNGA1–4, CNGB1, and CNGB3) have been identified in mammalian genomes. In contrast, the *Arabidopsis thaliana* (L.) Heynh. genome encodes 20 putative CNGC subunits that are thought to assemble in the same tetrameric form as animal subunits do. Although the study of *Arabidopsis* CNGCs is still in its infancy, current research revealed their ability to transport cations that play a role in mediating various biotic and abiotic stresses and developmental processes. This review discusses the current knowledge of plant CNGCs and focuses on functional aspects, with references to heterologous expression studies and reverse genetics analyses. In addition, structural aspects of these channels are discussed.

**Résumé :** Les canaux ioniques contrôlés par des nucléotides cycliques (CNGCs) constituent des canaux cationiques non sélectifs identifiés pour la première fois dans les neurones sensitifs des photorécepteurs de la rétine et de l'olfaction chez les vertébrés. On dispose d'une bonne documentation sur le rôle des CNGCs chez les animaux, et on croit qu'ils sont localisés sur la membrane plasmique comme hétérotétramères. Jusqu'ici on a identifié seulement six gènes des CNGCs (CNGCA1–4, CNGCB1 et 3) dans des génomes animaux. Au contraire, le génome de l'*Arabidopsis thaliana* code pour 20 présumées sous-unités de CNGC, que l'on croit s'assembler sous la même forme que les sous-unités animales le font. Bien que l'étude des CNGCs de l'*Arabidopsis* soit à ses débuts, la recherche en cours révèle leur capacité à transporter les cations jouant un rôle en intervenant dans divers stress biotiques et abiotiques ainsi que des processus développementaux. Dans cette revue, les auteurs discutent des connaissances actuelles sur les CNGCs des plantes et mettent l'accent sur les aspects fonctionnels, en relation avec des études d'expression hétérologues et des analyses génétiques inversées. On discute de plus les aspects structuraux de ces canaux.

[Traduit par la Rédaction]

## Physiological roles of cyclic-nucleotide monophosphates (cNMPs)

All organisms utilize networks of signaling pathways to elicit a plethora of biological responses involving developmental processes, abiotic and biotic stress responses, nutrient acquisition, and metabolic regulation. These networks employ a number of organic and inorganic molecules known as secondary messengers to convey information about a perceived stimulus to the cellular machinery required to generate a response (Bouché et al. 2005). These secondary messengers act on downstream effectors that ultimately initiate a multitude of cellular responses including gene expression, transport across membranes, and cytoskeletal reorganization (Bouché et al. 2005). One group of secondary messengers that have been found to play a prominent role in mammalian physiology and signal transduction are the cyclic-nucleotide monophosphates (cNMPs): 3′,5′-cyclic adenyl monophosphate (cAMP), and 3′,5′-cyclic guanyl monophosphate (cGMP) (Newton and Smith 2004; Kaplan et al. 2007; Rehmann et al. 2007; Martinez-Atienza et al. 2008). Cyclic AMP was first isolated in canine liver studies assessing the effect of epinephrine and glucagon on glycogen-phosphorylase activity (Rall et al. 1957). The discovery of cGMP soon followed and was found to be an important secondary messenger as well (Ashman et al. 1963; Rehmann et al. 2007). In animals, cAMP mediates the action of a wide range of hormones and neurotransmitters, and regulates an array of metabolic pathways (Newton and Smith 2004). Cy-

Received 14 September 2008. Published on the NRC Research Press Web site at botany.nrc.ca on 10 July 2009.

**K. Chin, W. Moeder, and K. Yoshioka.**[2] Department of Cell and Systems Biology, University of Toronto, 25 Willcocks Street, Toronto, ON M5S 3B2, Canada; Centre for the Analysis of Genome Evolution and Function (CAGEF), University of Toronto, 25 Willcocks Street, Toronto, ON M5S 3B2, Canada.

[1]This paper is one of a selection published in a Special Issue comprising papers presented at the 50th Annual Meeting of the Canadian Society of Plant Physiologists (CSPP) held at the University of Ottawa, Ontario, in June 2008.

[2]Corresponding author (e-mail: keiko.yoshioka@utoronto.ca).

clic GMP on the other hand, has a more restricted role, which includes the regulation of optic rod cells in response to light activation, smooth muscle contractions in response to nitric oxide (NO) (Moncada et al. 1992), and the movement of $Na^+$ and water across cell membranes (Hofmann et al. 1992; Newton and Smith 2004).

The physiological role of cNMPs in higher plants, however, is less understood and has been a fiercely debated topic for almost three decades. Cyclic NMPs have only recently been accepted as important secondary messengers in plants. These signaling molecules have been detected in several species including tobacco (*Nicotiana tabacum* L.), maize (*Zea mays* L.), and barley (*Hordeum vulgare* L.) (Richards et al. 2002; Newton and Smith 2004). While genes that encode for guanylate cyclases have been identified in *Arabidopsis* (Ludidi and Gehring 2003; Kwezi et al. 2007), only one report about a plant protein with adenylate cyclase activity has so far been published (Moutinho et al. 2001).

Both cGMP and cAMP are reported to be involved in a number of physiological processes (Talke et al. 2003; Kaplan et al. 2007; Martinez-Atienza et al. 2008). This includes phytochrome signaling where the antagonism of cGMP and $Ca^{2+}$ regulates chloroplast development and anthocyanin biosynthesis (Bowler et al. 1994*a*, 1994*b*). Cyclic NMPs have also been postulated to be involved in plant cell cycle progression. Ehsan et al. (1998, 1999) provided evidence of cAMP fluctuations during the cell cycle in tobacco BY-2 cells. Furthermore, they were able to inhibit cAMP biosynthesis at the beginning of the G1 phase with indomethacin and arrested cell cycle progression at the G1/S phase. cNMPs also act as secondary messengers in other developmental processes such as auxin-induced adventitious root growth and pollen tube growth (Moutinho et al. 2001; Pagnussat et al. 2003). Plant defense responses have also been shown to involve cNMPs. For instance, cAMP was reported to mediate defense responses in *Arabidopsis* to the pathogenic fungi *Verticillium dahliae* (Kleb., 1913) by regulating the production of salicylic acid (SA) (Jiang et al. 2005). In tobacco, cGMP can also mediate plant defense signaling downstream of NO by activating *pathogenesis-related 1* (*PR-1*) gene expression (Durner et al. 1998). Lastly, cNMPs have been shown to play a role in abiotic stress responses and ion homeostasis (Maathuis and Sanders 2001; Donaldson et al. 2004). For instance, the addition of cAMP or cGMP to *Arabidopsis* seedlings improves their salinity tolerance by negatively regulating voltage-independent channels in the root hairs (Maathuis and Sanders 2001). In addition, salt and osmotic stress rapidly increases cGMP levels in *Arabidopsis* (Donaldson et al. 2004).

Primary targets of cNMPs in animals are cyclic-nucleotide-dependent protein kinases (protein kinase A and protein kinase G; Rehmann et al. 2007). In plants, however, the existence of these targets remains elusive (Martinez-Atienza et al. 2008). Recently, a bioinformatics study by Bridges et al. (2005) identified several gene families with cyclic nucleotide binding domains (CNBDs). These include the Shaker-like $K^+$ channels (9 members in *Arabidopsis*), cyclic-nucleotide-regulated thioesterases (2 members in *Arabidopsis*) and cyclic-nucleotide-gated ion channels (CNGCs, 20 members in *Arabidopsis*). In this review, we focus on the current status of CNGC research in plants.

## Cyclic-nucleotide-gated ion channels (CNGCs)

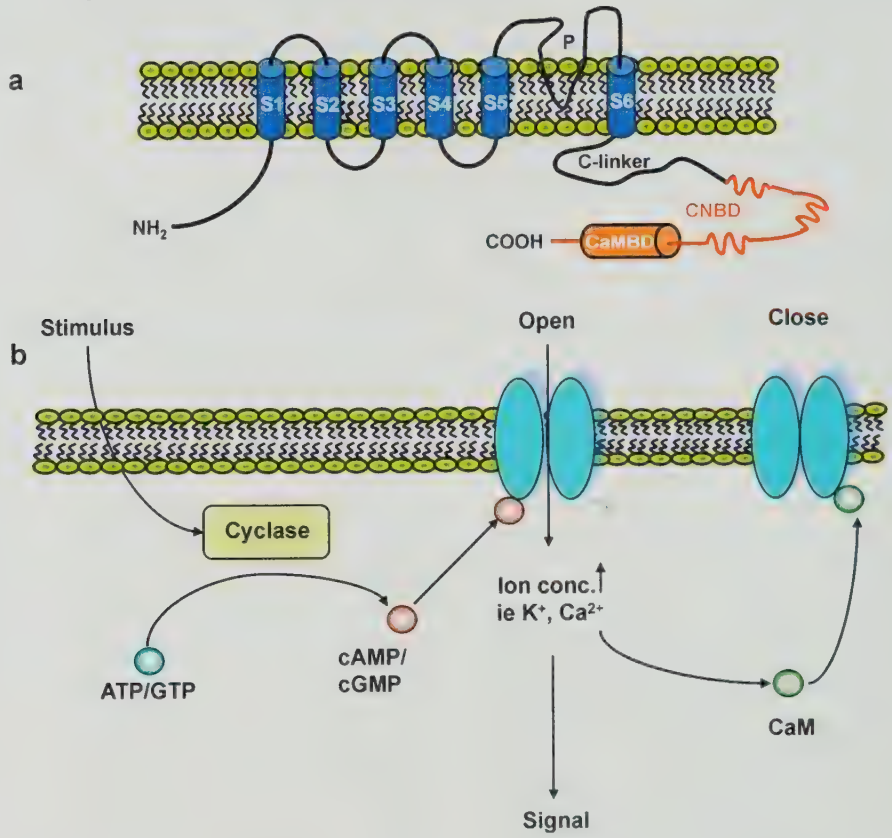
Ion influx is central to signal transduction, and one of the potential pathways for the uptake of these ions is via cyclic-nucleotide-gated ion channels (CNGCs) (Talke et al. 2003). CNGCs are nonselective cation channels that are gated by the direct binding of cyclic nucleotides, and were first identified in vertebrate visual and olfactory signal transduction cascades (Zagotta and Siegelbaum 1996; Craven and Zagotta 2006). CNGCs are composed of six transmembrane domains and a pore region between the fifth and sixth domains (Fig. 1*a*; Zagotta and Siegelbaum 1996; Hua et al. 2003*b*). The fourth transmembrane domain has similarities to the Shaker-type voltage sensor (Köhler et al. 1999; Rehmann et al. 2007). The N-terminal domain extends into the cytosol and is believed to bind calmodulin (CaM), while the C-terminal domain binds cNMPs. Plants, on the other hand, possess a slightly different structure where both the CaM and cyclic nucleotide binding domains occur in the cytosolic C-terminus in overlapping regions (Fig. 1*a*; Köhler and Neuhaus 2000; Hua et al. 2003*b*). The role of animal CNGCs in the signal transduction of light and odorant perception is well documented and they are believed to localize to the plasma membrane in heterotetramers (Kaupp and Seifert 2002; Talke et al. 2003). While six CNGC subunits (four α and two β) have so far been identified in mammalian genomes, the *Arabidopsis* genome encodes 20 putative CNGC subunits (Mäser et al. 2001) that are thought to assemble in the same heterotetrameric form as animal subunits (Hua et al. 2003*b*; Talke et al. 2003). The current model of CNGC signaling is summarized in Fig. 1*b*. It is based on research on animal CNGCs and involves the opening of the channel by either cAMP or cGMP, which leads to an influx of cations such as $K^+$ and $Ca^{2+}$. Binding of $Ca^{2+}$ activates CaM, allowing it to bind to the CaM-binding domain of the CNGC. This would close the channel, acting as a negative feedback mechanism.

Alignment of the 20 predicted amino-acid sequences categorized the members into four groups (I–IV), of which group IV is broken into two subgroups (IVA and IVB) (Mäser et al. 2001). Studies on some *Arabidopsis* CNGCs have so far revealed their ability to transport cations that play a role in mediating various environmental stresses, plant defense responses and development (Clough et al. 2000; Balagué et al. 2003; Chan et al. 2003; Gobert et al. 2006; Ma et al. 2006; Yoshioka et al. 2006; Borsics et al. 2007; summarized in Table 1). The details of their biological functions are discussed below.

## Ion selectivity

Traditionally, ion channel functional properties are assessed by electrophysiological methods using *Xenopus laevis* (Daudin, 1802) oocytes or human embryogenic kidney (HEK) cells. Some CNGCs have been functionally characterized by analyzing channel currents in voltage clamp studies. In 1999, Leng et al. demonstrated that the expression of *AtCNGC2* in *Xenopus laevis* oocytes resulted in cNMP-dependent $K^+$ currents in the presence of either lipophilic cAMP or cGMP. They further confirmed AtCNGC2 to be an inward-rectified cNMP-gated ion channel. Using guard cell plasma membranes, Ali et al. (2007) demonstrated (by patch clamp analysis) cAMP-activated currents in

**Fig. 1.** (*a*) Structure of plant cyclic-nucleotide-gated ion channels (CNGC). CNGCs are composed of six transmembrane domains (S) and a pore region (P) between the fifth and sixth domains. The cytosolic C-terminus has a C-linker followed by the cyclic nucleotide binding domain (CNBD) with an overlapping calmodulin binding domain (CAMBD). (*b*) Model of CNGC-mediated signaling in plants. Upon perception of a stimulus, adenylate or guanylate cyclase produce cAMP or cGMP, respectively, which bind to the CNBD. Binding causes a conformational change leading to opening of the channel. This causes an influx of monovalent and divalent cations in the cell. This elevation in cation concentration activates downstream signaling components that result in a physiological response. $Ca^{2+}$-activated calmodulin (CaM) binding leads to closing of the CNGC as a negative feedback mechanism.




membranes from wild type but not *dnd1* mutants, which lack *AtCNGC2* function. A report using *X. laevis* oocytes suggested that AtCNGC2 may also be selective for other monovalent cations ($Li^+$, $Cs^+$, $Rb^+$) to an extent nearly as great as $K^+$ (Leng et al. 2002). Interestingly, expression of *AtCNGC2* in both *X. laevis* oocytes and HEK cells showed a strong discrimination against $Na^+$ (Leng et al. 2002). In contrast, AtCNGC1 was found to be an inward rectified ion channel equally permeable to both $K^+$ and $Na^+$ (Hua et al. 2003*a*). It should be noted that the amino acid triplet motif GYG of the $K^+$ selectivity filters in $K^+$ selective channels has been demonstrated to be a strong basis for discrimination against $Na^+$ conductivity (Leng et al. 2002). Animal CNGCs, on the other hand, lack this motif. Instead, they possess a GET motif and have been shown to be nonselective for divalent and monovalent cations, and do not discriminate between $K^+$, $Na^+$, and $Ca^{2+}$ (Kaplan et al. 2007). Plant CNGCs however, do not contain the GYG or GET motifs (conserved in animals) (Hua et al. 2003*a*). Through computational modeling, Hua et al. (2003*a*) showed that AtCNGC1 and AtCNGC2 have a GQN and AND triplet, respectively. A GQN or similar selectivity filter motif is present in 16 out of 20 *Arabidopsis* CNGCs (Kaplan et al. 2007), while the AND motif was unique to only AtCNGC2. They further showed that by changing the AND filter of AtCNGC2 to AET, which is more closely related to animal CNGCs, $Na^+$ conductance is recovered in both *X. laevis* oocytes and HEK cells (Hua et al. 2003*a*). It was thus suggested that AtCNGC2's AND motif facilitates $K^+$ conductance and $Na^+$ exclusion in a fashion similar to the GYG triplet of $K^+$ Shaker-like channels (Hua et al. 2003*a*). In the same year, Balagué et al. (2003) demonstrated that *X. laevis* oocytes expressing *AtCNGC4* showed both $K^+$ and $Na^+$ outward rectified currents that are activated by both cAMP and cGMP, with the latter being more efficient. Additionally, these currents are blocked by $Cs^+$ ions. Like AtCNGC2, the selectivity filter of AtCNGC4 is believed to differ from the rest of the family members and is exclusive to AtCNGC4. Very recently, expression of *AtCNGC10* in HEK cells was also shown to conduct inward $K^+$ currents that are activated by lipophilic cGMP (Christopher et al. 2007).

So far only a few plant CNGCs have been characterized

**Table 1.** Ion selectivities and physiological roles identified in plant CNGCs.

| Gene | Gene ID | Ion selectivity | Suggested physiological role | Reference |
|---|---|---|---|---|
| ***Arabidopsis thaliana*** | | | | |
| *AtCNGC1* | AT5G53130 | $K^+$, $Na^+$, $Ca^{2+}$, $Pb^{2+}$ | Cation uptake from soil | Hua et al. 2003*a*; Mercier et al. 2004 |
| | | | Heavy metal uptake | Sunkar et al. 2000; Ali et al. 2005 |
| *AtCNGC2* | AT5G15410 | $K^+$, $Ca^{2+}$, $Li^{2+}$, $Cs^+$, $Rb^+$ | Developmental cell death and senescence | Leng et al. 1999, 2002; Mercier et al. 2004 |
| | | | Growth and development | Köhler et al. 2001; Chan et al. 2003 |
| | | | Pathogen resistance | Ali et al. 2007; Clough et al. 2000 |
| *AtCNGC3* | AT2G46430 | $K^+$, $Na^+$ | Distribute and translocate ions from xylem | Gobert et al. 2006 |
| | | | Translocate $Na^+$ within embryo | |
| *AtCNGC4* | AT5G54250 | $K^+$, $Na^+$ | Pathogen resistance | Mercier et al. 2004; Balagué et al. 2003; Jurkowski et al. 2004 |
| *AtCNGC10* | AT1G01340 | $K^+$ | Light modulated development | Li et al. 2005; Borsics et al. 2007; Christopher et al. 2007 |
| *AtCNGC11* | AT2G46440 | $K^+$, $Ca^{2+}$ | Pathogen resistance | Yoshioka et al. 2006; Urquhart et al. 2007 |
| *AtCNGC12* | AT2G46450 | $K^+$, $Ca^{2+}$ | Pathogen resistance | Yoshioka et al. 2006; Urquhart et al. 2007 |
| *AtCNGC18* | AT5G14870 | $Ca^{2+}$ | Polarized pollen tube growth | Frietsch et al. 2007 |
| ***Nicotiana tabacum*** | | | | |
| *NtCPB4* | AF079872 | $Pb^{2+}$ | Heavy metal uptake | Arazi et al. 1999 |
| ***Hordeum vulgare*** | | | | |
| *HvCBT1* | AJ002610 | Unknown | Ion transport in aleurone | Schuurink et al. 1998 |
| *NEC1* | AY972627 | Unknown | Pathogen resistance | Rostoks et al. 2006 |

by electrophysiological methods. This has been partially attributed to the instability of plant CNGCs in these cells as reported by Leng et al. (1999) and Balagué et al. (2003). Additionally, it requires specialized skills and costly infrastructure. Thus, in recent years the functional properties of plant CNGCs have been characterized through the use of heterologous systems involving cation-uptake deficient yeast (*Saccharomyces cerevisiae*) and *Escherichia coli* mutants.

One commonly used yeast mutant is the CY162 strain, which lacks two major $K^+$ transporters, *TRK* and *TRK2*, and cannot survive in low-$K^+$ media without a complementing channel (Ko and Gaber 1991). In 1998, Schuurink et al. demonstrated that the barley CNGC, HvCBT1, cannot complement the mutant on low-$K^+$ media. Hydropathy analysis suggested that HvCBT1 possesses six transmembrane domains similar to those of $K^+$ Shaker-type channels and animal CNGCs, but with a GQN instead of the conserved GYG consensus sequence of $K^+$ selective channels in the pore region. By changing the pore to the GYG consensus sequence, yeast growth is further attenuated (Schuurink et al. 1998). In a similar assay, the expression of *AtCNGC10* is able to rescue yeast growth (Li et al. 2005), suggesting that AtCNGC10 can form $K^+$ permeable channels in yeast. Leng et al. (1999) demonstrated that the *Arabidopsis* AtCNGC2 partially complements the CY162 yeast mutant in the presence of membrane-permeable (dibutyryl)-cAMP or cGMP when grown on solid and liquid media with low $K^+$. AtCNGC3 also partially complements a similar yeast strain deficient in the TRK1, TRK2, and TOK1 $K^+$ transporters (Gobert et al. 2006). In a slightly modified analysis, Mercier et al. (2004) later demonstrated the ability of AtCNGC1, AtCNGC2, and AtCNGC4 to complement the hypersensitivity of the *trk1*/*trk2*-deficient WD3 yeast mutant to the cationic amino glycoside, hygromycin B, in low $K^+$. The expression of *AtCNGC1*, *AtCNGC2*, and *AtCNGC4* was shown to further enhance yeast growth in the presence of dibutyryl-cAMP. Using a similar protocol, Yoshioka et al. (2006) reported the complementation of the WD3 mutant with AtCNGC11, AtCNGC12, and the chimeric AtCNGC11/12. However, contrary to the finding of Leng et al. (1999) on AtCNGC2, the expression of *AtCNGC11*, *AtCNGC12*, and *AtCNGC11/12* exhibits enhanced yeast growth in the presence of only lipophilic cAMP, suggesting that these channels are activated solely by cAMP and not cGMP. More recently, the same analysis incorporated into a structure–function study of AtCNGC11/12 demonstrated the physical binding of cAMP to AtCNGC12 and AtCNGC11/12, further supporting this notion (Baxter et al. 2008).

CNGCs have also been functionally characterized with the use of yeast $Ca^{2+}$- and $Na^+$-channel mutants. Ali et al. (2005) utilized a $Ca^{2+}$ uptake-deficient mutant lacking the $Ca^{2+}$ transporters MID1 and CCH1 (ELY151). These transporters are involved in the response to α mating factor and the mutant strain exhibits growth arrest in the presence of this factor. Ali et al. (2005) demonstrated the ability of AtCNGC1 to complement the yeast mutant strain. The same assay was employed by Urquhart et al. (2007) to show that AtCNGC11, AtCNGC12, and AtCNGC11/12 also can conduct $Ca^{2+}$ in yeast. Gobert et al. (2006) assessed $Na^+$ uptake of AtCNGC3 by using the salt-sensitive G-19 yeast mutant strain that carries deletions in the ENA1–4 $Na^+$-extruding pumps. Mutant yeast expressing *AtCNGC3* are hypersensitive to high salt concentrations and accumulate significantly higher amounts of $Na^+$ compared with the empty vector control, indicating that AtCNGC3 can act as $Na^+$ channels in yeast (Gobert et al. 2006).

The $K^+$-uptake-deficient *E. coli* strain LB650 with mutations in the *TRK H* and *TRK G* genes has also been used to assess the $K^+$ channeling of some CNGCs. Ali et al. (2005) demonstrated that AtCNGC1 can also rescue bacterial growth in this system. In the same year, Li et al. (2005) reported similar results when *AtCNGC10* is expressed in LB650. A summary of the ion selectivity of each CNGC is listed in Table 1. However, as Kaplan et al. (2007) also pointed out, it is not completely clear how well these heterologous expression systems reflect their in vivo function. Therefore, careful interpretation of these results and further assessment are required.

**CNGC transgenics and mutants exhibit both cation tolerance and sensitivity**

The study of different cations on the growth of *Arabidopsis* CNGC mutants has been useful in elucidating the physiological roles of these channels in planta. Arazi et al. (1999) first reported that the overexpression of a tobacco plasma membrane CNGC designated *NtCBP4* conferred tolerance to $Ni^{2+}$ and hypersensitivity to $Pb^{2+}$. A later study demonstrated that transgenic tobacco plants expressing a truncated version of *NtCBP4* has improved tolerance to $Pb^{2+}$ and attenuated $Pb^{2+}$ accumulation compared to those expressing the full-length gene (Sunkar et al. 2000). Furthermore, T-DNA insertional mutants of *AtCNGC1*, which encodes the homologous protein in *Arabidopsis*, exhibit $Pb^{2+}$ tolerance, as well as significantly lower levels of $Pb^{2+}$ accumulation (Sunkar et al. 2000). Unlike its tobacco counterpart, this tolerant phenotype is not observed with $Ni^{2+}$, suggesting that AtCNGC1 elicits a response that is specific to $Pb^{2+}$. Taken together, these results indicate that NtCPB4 and AtCNGC1 are likely involved in heavy metal uptake across the plasma membrane. More recently, Ma et al. (2006) demonstrated that the null mutation of *AtCNGC1* reduces $Ca^{2+}$ uptake in similar magnitude to the reduction in $Pb^{2+}$ uptake reported by Sunkar et al. (2000). This suggests that AtCNGC1 is similarly permeable to both $Ca^{2+}$ and $Pb^{2+}$. Since *AtCNGC1* seems to be expressed primarily in *Arabidopsis* roots (Ma et al. 2006), it may be involved in ion uptake from the soil.

Two mutant alleles of *AtCNGC2* have been tested for sensitivity to pH changes, $Mg^{2+}$, $K^+$, $Na^+$ and $Ca^{2+}$. Both null mutants are exclusively hypersensitive to $Ca^{2+}$ (Chan et al. 2003). When compared to their wild type counterparts, the mutant plants have smaller rosette sizes, root mass and overall stature (Chan et al. 2003). Preliminary results showed that the $Ca^{2+}$ hypersensitivity of *cngc2* mutants is not caused by the accumulation of $Ca^{2+}$, but rather by a defective signaling pathway in the high $Ca^{2+}$ environment. Genome wide transcriptional analysis of *cngc2* and wild type plants under normal and elevated $Ca^{2+}$ conditions revealed that the transcriptional profile of *cngc2* plants under normal conditions is similar to that of wild type plants under high $Ca^{2+}$ condition (Chan et al. 2008). These results indicate that the role of AtCNGC2 may not be $Ca^{2+}$uptake, but rather signal transduction and homeostasis. Interestingly, hierarchical clustering revealed similarities in expression patterns between *cngc2* plants under high $Ca^{2+}$ and various biotic and abiotic stresses, suggesting the involvement of $Ca^{2+}$/CNGCs in the signal transduction during these stress responses (Chan et al. 2008).

*AtCNGC3* has been recently found to be also involved in ion homeostasis in planta. Gobert et al. (2006) reported that *cngc3* null mutants exhibit impaired germination in response to high external levels of $Na^+$. When compared with wild type, the three allelic mutant lines have significantly less germination in the presence of high external $Na^+$, but not $K^+$ or $NH_4^+$ (Gobert et al. 2006). Furthermore, isomolar concentrations of sorbitol have a similar effect on mutant and wild type plants, suggesting that the impaired germination is caused by specific sensitivity to $Na^+$ (Gobert et al. 2006). In addition, *cngc3* mutant plants grow better than wild type when exposed to intermediate concentrations of NaCl, whereas no discernible differences are observed with exposure to $Zn^{2+}$, $Pb^{2+}$, $Cd^{2+}$, $Ca^{2+}$, or sorbitol. Measurements of ion levels within tissue revealed that *cngc3* and wild type seedlings have similar $Na^+$ concentations but significantly lower $K^+$ concentrations in *cngc3*, indicating that the mutant plants are capable of preventing $K^+$ uptake (Gobert et al. 2006). This suggested that AtCNGC3 does not play a prominent role in salinity adaptation. GUS reporter gene analysis revealed *AtCNGC3* expression to be prevalent in the embryo, leaf and root tissue throughout development and it has been suggested that AtCNGC3 plays a role in the distribution and translocation of ions from the xylem. Furthermore, germination results indicated that AtCNGC3 does not participate in ion uptake during germination, but rather is involved in transporting toxic levels of $Na^+$ from sensitive to tolerant tissues within the embryo (Gobert et al. 2006). In addition, it was also suggested that AtCNGC3 is part of a $Na^+$ uptake pathway during seedling development where a null mutation results in less accumulation of $Na^+$ (Gobert et al. 2006).

Recently, it was demonstrated that *AtCNGC11* and *AtCNGC12* are involved in ion homeostasis as well (Chin et al. 2008). Root growth experiments revealed that single knockout mutants for *AtCNGC11* or *AtCNGC12* are similarly sensitive as wild type to elevated levels of $Ca^{2+}$ and $K^+$. In contrast, a potential double knockout of *AtCNGC11* and *AtCNGC12* have enhanced sensitivity to only $Ca^{2+}$, but not $K^+$ (K. Chin and K. Yoshioka, unpublished data), suggesting that the elimination of both genes might inadvertently cause the cell to overcompensate for their loss by upregulating other $Ca^{2+}$ ion channels. Furthermore, these results suggest potential redundancy in the biological function of both genes (Chin et al. 2008). Further analyses are in progress to draw conclusions on the role of *AtCNGC11* and *AtCNGC12* in ion homeostasis.

**The role of CNGCs in plant development**

The ion sensitivities and expression profile of different CNGCs reported above suggests that these ion channels play a prominent role in regulating various developmental pathways. GUS reporter gene analysis revealed the expression of *AtCNGC2* to be prominent in cotyledons of 14-day-old seedlings, and in leaves of flowering plants (Köhler et al. 2001). Köhler et al. also reported that *AtCNGC2* expression is induced during the early stages of plant senescence, as well as in the dehiscence zone of mature siliques. Therefore, these results suggested that *AtCNGC2* is a positive regulator of the developmental cell death program that is expressed throughout the life cycle of *Arabidopsis* plants,

starting from early seedling development up until the early stages of senescence (Köhler et al. 2001). More recently, the hypersensitive phenotype of *cngc2* mutants to $Ca^{2+}$ has been hypothesized to result in severe reductions in plant size and fertility, again suggesting that *AtCNGC2* is likely involved in certain developmental processes (Chan et al. 2003).

Borsics et al. (2007) demonstrated that *AtCNGC10* antisense lines exhibit significant changes in growth and metabolism. Compared with wild type plants, the antisense lines have a 20% reduction in leaf surface area, laminar thickness, and palisade columnar cell length (Borsics et al. 2007). Furthermore, the leaves of the antisense plants accumulate almost double the amount of starch than wild type, and flowering occurs approximately 10 d earlier. The root lengths in the antisense seedlings are also significantly shorter than wild type, and exhibit impaired gravitropic responses (Borsics et al. 2007). Taken together, it was suggested that *AtCNGC10* is involved in a multitude of developmental pathways involving light modulation because of the similarities in phenotype to weak photoreceptor mutants (Weston et al. 2000; Chun et al. 2001; Borsics et al. 2007). The inability to generate homozygous T-DNA insertion mutant lines and a low frequency of antisense plant recovery further support the notion that *AtCNGC10* is an essential gene with very low redundancy.

Recently, Frietsch et al. (2007) investigated the role of *AtCNGC18* in pollen tube development. GUS reporter gene analysis revealed that *AtCNGC18* is primarily expressed in pollen grains, and not in the leaves or roots of young seedlings (Frietsch et al. 2007). Analysis of the pollen tubes from two allelic *cngc18* mutants showed that they develop into short and thin tubes that grow nondirectionally for a short distance before premature termination, resulting in male sterility (Frietsch et al. 2007). Heterologous expression of *AtCNGC18* in *E. coli* results in an accumulation of $Ca^{2+}$, suggesting that *AtCNGC18* transduces a signal that regulates pollen-tube growth. Interestingly, Moutinho et al. (2001) previously showed that cAMP acts as a signal for pollen tube growth and reorientation. In a study of transporter genes that are expressed during pollen development, *AtCNGC7, 8, 16* and *18* were identified to be primarily expressed in pollen (Bock et al. 2006), indicating the importance of CNGCs in pollen development. Therefore, it is very likely that further specific roles of CNGCs during plant development will be elucidated.

**Involvement of CNGCs in pathogen defense responses**

A number of CNGCs have been implicated in mediating pathogen defense responses in plants. Yoshioka et al. (2001) identified the *cpr22* mutant (*constitutive expresser of PR genes* 22) that is generated by a 3 kb deletion that fuses the 5′ portion of *AtCNGC11* and the 3′ portion of *AtCNGC12* to form the chimeric *AtCNGC11/12* gene. This mutation confers a stunted morphology with curly leaves, hypersensitive response (HR)-like spontaneous lesion formation in leaves, SA accumulation, and enhanced disease resistance to oomycete and bacterial pathogens (Yoshioka et al. 2001). Subsequent work suggested that *AtCNGC11* and *AtCNGC12* are positive regulators of pathogen defense signaling (Yoshioka et al. 2006). *AtCNGC11* and *AtCNGC12* knockout mutants exhibit partial breakdown of resistance to infection by the avirulent *Hyaloperonospora parasitica*, isolate Emwa1, compared with wild type plants (Yoshioka et al. 2006).

The *cpr22* phenotype is not caused by the absence of either channel, *AtCNGC11* or *12*, but rather by the expression of the chimeric *AtCNGC11/12* (Yoshioka et al. 2006). Furthermore, the requirement of functionally active AtCNGC11/12 for the *cpr22* phenotype was also confirmed (Baxter et al. 2008). AtCNGC11/12, like its wild type proteins, AtCNGC11 and AtCNGC12, conducts both $Ca^{2+}$ and $K^+$. In yeast complementation assays no difference in ion conductance efficiency was found between AtCNGC11/12 and its wild type proteins. However, the $Ca^{2+}$ channel blockers, $Gd^{3+}$ and $La^{3+}$, but not the $K^+$ channel blocker tetraethylammonium chloride (TEA) suppress AtCNGC11/12-induced HR-like cell death formation (Urquhart et al. 2007), indicating that $Ca^{2+}$ influx is required for HR-like cell death formation and is at least in part mediated by CNGCs. Urquhart et al. (2007) also showed that the cell death induced by AtCNGC11/12 is not due to unspecific perturbation of cellular physiology. Molecular and microscopic analyses revealed that AtCNGC11/12 activates programmed cell death (PCD) in plants. Furthermore, caspase-like activity is required for AtCNGC11/12-induced PCD. Caspases are cystein proteases that have been shown to regulate PCD in animals. There are no true orthologues of caspases in plants, but the best candidates for caspase-like proteins in plants so far are the vacuolar processing enzymes (VPE; Hatsugai et al. 2006). Both treatment with caspase-1 inhibitors (Ac-YVAD-CHO and Ac-YVADCMK) and silencing of *VPE1a* and *VPE1b* strongly attenuates AtCNGC11/12-induced cell death (Urquhart et al. 2007).

Other defense-related mutants are *dnd1* and *dnd2/hlm1*. They also have been shown to exhibit spontaneous cell death formation and broad-spectrum disease resistance (Clough et al. 2000; Balagué et al. 2003; Jurkowski et al. 2004). The barley *nec1* mutation, which induces necrotic lesions, was also mapped to the homolog of *DND2/HLM1* in barley (Rostoks et al. 2006). Both *dnd1* and *dnd2 /hlm1* have mutations that destroy channel function of AtCNGC2 and AtCNGC4, respectively (null mutations). They exhibit constitutive *pathogenesis-related* (*PR*) gene expression, elevated levels of SA, and enhanced disease resistance to virulent pathogens similar to *cpr22* (Clough et al. 2000; Balagué et al. 2003; Jurkowski et al. 2004). Furthermore, the *dnd* mutants maintain their gene-for-gene resistance to some avirulent pathogens even though HR formation after infection is attenuated (Yu et al. 1998; Clough et al. 2000; Balagué et al. 2003). Interestingly, as described above activation of resistance responses such as SA accumulation and *PR* gene expression seen in *cpr22*, are induced by expression of *AtCNGC11/12* and by the lack of *AtCNGC2* or *AtCNGC4* in *dnd1* and *dnd2/hlm1*, respectively. This suggests that a precise cation signal/homeostasis is required to activate PCD.

More recently, electrophysiological analysis by Ali et al. (2007) demonstrated that *AtCNGC2* transduces a $Ca^{2+}$ current that leads to an increase in NO production. As mentioned above, the *dnd1* mutant does not display HR after infection with some avirulent pathogens. However, exoge-

nous NO treatment can restore HR formation indicating that NO formation is downstream of $Ca^{2+}$ influx. (Ali et al. 2007) It is known that NO synthase (NOS) in animals is a $Ca^{2+}$/CaM dependent enzyme. Recently, Ma et al. (2008) provided some evidence that CaM-like 24 (CML24) is required for $Ca^{2+}$ -dependent activation of plant NOS as well. As described in the previous section, AtCNGC11/12 induces HR-like PCD in a $Ca^{2+}$-dependent manner (Urquhart et al. 2007). These data suggest a tight connection of CNGCs and $Ca^{2+}$ with pathogen resistance signal transduction. A number of studies have reported the importance of $Ca^{2+}$ influx in pathogen resistance responses. However, so far concrete components/channels to regulate $Ca^{2+}$ fluxes after pathogen infections have not been clearly characterized. Thus, the study of CNGCs in pathogen resistance may be a novel avenue for studying $Ca^{2+}$ signal transduction in plant immune responses.

**Structural analysis of molecular composition of CNGCs**

Although structure–function analyses of plant CNGCs are still at its infancy, CNGCs in animals have been relatively well studied and documented. The structure of the cytoplasmic C-terminal region including the CNBD and the C-linker domains of a hyperpolarization-activated cyclic-nucleotide-gated channel 2 (HCN2) was recently solved by X-ray crystallography (Zagotta et al. 2003). The HCN channels belong to a superfamily of voltage-gated channels that play an essential role in relaying electrical signals involved in cardiac and neuronal pacemaker activities in animals (Zagotta et al. 2003). In addition, these channels are similar to those of the CNGC, eag- (ether a go-go), and KAT1-related Shaker-type $K^+$ channels (Zagotta et al. 2003). The C-terminal region possesses a tetrameric configuration with the CNBD composed of four α-helices and a β-roll in which cyclic nucleotides bind and interact with the C-linker. Furthermore, the CNBD of HCN2 has a similar fold to the CNBD of the *E. coli* catabolite gene activator protein (CAP) and the regulatory unit of the cAMP dependent protein kinase A. In addition, HCN2 is specifically regulated by cAMP even though cGMP interactions can occur. It was postulated that cAMP binding causes a conformational change in the CNBD that is then coupled with a conformational change in the C-linker, leading to the opening of the channel (Zagotta et al. 2003). It has been shown that CNGCs likely function as heterotetramers in animals (Zheng and Zagotta 2004; Craven and Zagotta 2006), but whether this also is true in plants is still unknown. Furthermore, knowledge of the inter- and intra-subunit interactions in plants is also absent. In animals, the cytosolic N-terminus of the olfactory CNGA2 channel subunit interacts either with its own or with its partner's C-terminus, and this interaction is necessary to promote channel opening through stabilizing the conformations of the C-linker and the CNBD (Varnum and Zagotta 1997; Kaupp and Seifert 2002; Trudeau and Zagotta 2003). In addition, similar interactions have been reported to occur between the cytoplasmic N- and C-terminal regions of the animal photoreceptor rod subunits CNGA1 and CNGB1 (Varnum and Zagotta 1997; Kaupp and Seifert 2002; Trudeau and Zagotta 2003).

In light of the large number of CNGCs in plants, the possible combinations that can form functional hetero/homomeric channels are immense. Thus, the identification of all the interacting subunit partners remains incredibly challenging. So far, no crystal structure of a plant CNGC has been reported. Recently, Hua et al. (2003*b*) modeled the CNBD belonging to AtCNGC2. Using the previously solved structure of the cAMP dependent protein kinase A, RIα, it was shown that cAMP resides in the β barrel in the syn-conformation in AtCNGC2, which is different from the anti-conformation seen in animals (Hua et al. 2003*b*; Zagotta et al. 2003). Another notable difference from animal CNGCs is the bifunctionality of the CNBD. Through the identification of structural similarities between the truncated CNBD of RIα (domain B) and the CNBD of AtCNGC2, Hua et al. (2003*b*) suggested that plant CNGCs may have permitted the evolution of a CaM binding domain in this region while still retaining full cyclic nucleotide binding ability. This feature so far seems to be unique to plant CNGCs, as shown by previous work from Köhler and colleagues (Köhler et al. 1999; Köhler and Neuhaus 2000). More recently, Bridges et al. (2005) generated an in silico model through the alignment of the CNBDs of AtCNGC2, AtCNTE1, and AtKAT1 with the previously solved structures of PKA, Epac1, Epac2, CGK2, HCN2 and CAP. They were able to show that *At*CNTE1, the plant protein that possesses the highest amount of similarity to known crystal structures, models very well against all CNBDs with a high level of conservation of all secondary structures (Bridges et al. 2005). Furthermore, phylogenetic analyses of the plant CNBDs suggested they are classed into three subfamilies (CNTEs, CNGCs, and Shaker-type channels) that completely mirror the phylogeny of full-length proteins. This suggested that the CNBDs arose prior to isoform duplication and their commonality to both *Arabidopsis* and rice further indicated that plant specific nucleotide responses likely developed prior to the monocot–dicot divergence (Bridges et al. 2005).

Detailed mutation studies of functional domains in CNGCs have also emerged to elucidate the structural and regulatory properties of these channels. Baxter et al. (2008) identified a functionally essential amino acid for CNGCs by utilizing an intragenic suppressor mutant of *cpr22* (*AtCNGC11/12*). Computational modeling and in vitro cAMP-binding assays indicated that E519 in AtCNGC11/12 (and also the equivalent residue in AtCNGC12) is a key residue for the structural stability and functionality of AtCNGCs through the interaction of the C-linker and the CNBD (Fig. 1*a*; Baxter et al. 2008). Interestingly, E519 aligns well to Y573 in human CNGA3 where a mutation in this residue causes achromatopsia (Wissinger et al. 2001). Furthermore, the Y573 residue is conserved among various organisms, suggesting that this amino acid position plays a functionally important role for CNGCs in general.

## Concluding remarks

The completion of the *Arabidopsis* genome-sequencing project revealed a large family of CNGCs composed of 20 members with overall sequence similarities ranging between 55% and 83% (Mäser et al. 2001). While mammalian genomes have so far been found to encode six CNGC encoding genes, the large number and diversity of their plant

counterparts suggests important and perhaps very specialized physiological roles of CNGCs in plants. To elucidate this point, the analysis of expression patterns of the different CNGCs will be useful. Indeed, publicly available microarray data suggest specific functions and tissue specificity for individual CNGCs or groups (Talke et al. 2003; Bock et al. 2006). However, for complete understanding of the function of individual CNGC genes, much more detailed analyses are required. In addition, some functions of CNGCs may be difficult to uncover due to potential redundancy of the large number of CNGCs in plants. Furthermore, it is not clear whether the loss of one subunit will render any partnering subunits inactive or if the remaining subunits will form novel channels with new partners. The latter would thus create alternate phenotypes that are not directly related to the function of the missing subunit. Therefore, the key to understanding CNGC function, as well as regulation, is to find out which subunits interact with each other and whether these interactions are "permanent" or whether the subunits rearrange depending on the stimulus or the developmental stage. For such analyses, further technological breakthroughs that allow the monitoring of spatial and temporal interactions between subunits will be required.

As we discussed in this review, although only a handful of plant CNGCs have been investigated so far, it seems apparent that CNGCs play important roles for plant survival and adaptation by mediating multiple stress responses and developmental pathways. The molecular mechanism and downstream signaling components of $Ca^{2+}$ mediated signal transduction in animals is well studied. However, in plants, although numerous studies have shown the importance of $Ca^{2+}$ in many signaling pathways, much remains unknown. It has been suggested that CNGCs and glutamate receptors are prime candidates to facilitate the $Ca^{2+}$ influxes required for many processes (White et al. 2002). Thus, the study of CNGCs is required to understand $Ca^{2+}$ signal transduction in plants. Furthermore CNGCs can be used as a tool to identify downstream components of $Ca^{2+}$-mediated signaling in plants. Further efforts to characterize plant CNGCs is expected to fill in some of these gaps.

## Acknowledgements

We would like to thank to Mr. William Urquhart and Mr. Steve Mosher for proof reading this manuscript. We also would like to thank the University of Toronto for support to K.C. This work was supported by a Discovery grant of the Natural Sciences and Engineering Research Council of Canada, Canadian Foundation for Innovation, the Ontario Research Fund, and the Early Researcher Award of the Ontario Ministry of Research and Innovation to K.Y.

## References

Ali, R., Zielinski, R.E., and Berkowitz, G.A. 2005. Expression of plant cyclic nucleotide-gated cation channels in yeast. J. Exp. Bot. **57**: 125–138. doi:10.1093/jxb/erj012. PMID:16317039.

Ali, R., Ma, W., Lemtiri-Chlieh, F., Tsaltas, D., Leng, Q., Von Bodman, S., and Berkowitz, G.A. 2007. Death don't have no mercy and neither does calcium: *Arabidopsis* cyclic nucleotide gated channel 2 and innate immunity. Plant Cell, **19**: 1081–1095. doi:10.1105/tpc.106.045096. PMID:17384171.

Arazi, T., Sunkar, R., Kaplan, B., and Fromm, H. 1999. A tobacco plasma membrane calmodulin-binding transporter confers $Ni^{2+}$ tolerance and $Pb^{2+}$ hypersensitivity in transgenic plants. Plant J. **20**: 171–182. doi:10.1046/j.1365-313x.1999.00588.x. PMID: 10571877.

Ashman, D.F., Lipton, R., Melicow, M.M., and Price, T.D. 1963. Isolation of adenosine 3′,5′-monophosphate and guanosine3′,5′-monophosphate from rat urine. Biochem. Biophys. Res. Commun. **11**: 330–334. doi:10.1016/0006-291X(63)90566-7. PMID: 13965190.

Balagué, C., Lin, B.Q., Alcon, C., Flottes, G., Malmstrom, S., Köhler, C., Neuhaus, G., Pelletier, G., Gaymard, F., and Roby, D. 2003. HLM1, an essential signaling component in the hypersensitive response, is a member of the cyclic nucleotide-gated channel ion channel family. Plant Cell, **15**: 365–379. doi:10.1105/tpc.006999. PMID:12566578.

Baxter, J., Moeder, W., Urquhart, W., Shahinas, D., Chin, K., Christendat, D., Kang, H.G., Angelova, M., Kato, N., and Yoshioka, K. 2008. Identification of a functionally essential amino acid for *Arabidopsis* cyclic nucleotide gated ion channels using the chimeric *AtCNGC11/12* gene. Plant J. **56**: 457–469. PMID:18643993.

Bock, K.W., Honys, D., Ward, J.M., Padmanaban, S., Nawrocki, E.P., Hirschi, K.D., Twell, D., and Sze, H. 2006. Integrating membrane transport with male gametophyte development and function through transcriptomics. Plant Physiol. **140**: 1151–1168. doi:10.1104/pp.105.074708. PMID:16607029.

Borsics, T., Webb, D., Andeme-Ondzighi, C., Staehelin, L.A., and Christopher, D.A. 2007. The cyclic nucleotide-gated calmodulin-binding channel AtCNGC10 localizes to the plasma membrane and influences numerous growth responses and starch accumulation in *Arabidopsis thaliana*. Planta, **225**: 563–573. doi:10.1007/s00425-006-0372-3. PMID:16944199.

Bouché, N., Yellin, A., Snedden, W.A., and Fromm, H. 2005. Plant-specific calmodulin-binding proteins. Annu. Rev. Plant Biol. **56**: 435–466. doi:10.1146/annurev.arplant.56.032604.144224. PMID:15862103.

Bowler, C., Neuhaus, G., Yamagata, H., and Chua, N.H. 1994*a*. Cyclic GMP and calcium mediate phytochrome phototransduction. Cell, **77**: 73–81. doi:10.1016/0092-8674(94)90236-4. PMID:8156599.

Bowler, C., Yamagata, H., Neuhaus, G., and Chua, N.H. 1994*b*. Phytochrome signal transduction pathways are regulated by reciprocal control mechanisms. Genes Dev. **8**: 2188–2202. doi:10.1101/gad.8.18.2188. PMID:7958888.

Bridges, D., Fraser, M.E., and Moorhead, G.B.G. 2005. Cyclic nucleotide binding proteins in the *Arabidopsis thaliana* and *Oryza sativa* genomes. BMC Bioinformatics, **6**(6): 6. doi:10.1186/1471-2105-6-6.

Chan, C.W.M., Schorrak, L.M., Smith, R.K., Bent, A.F., and Sussman, M.R. 2003. A cyclic nucleotide-gated ion channel, CNGC2, is crucial for plant development and adaptation to calcium stress. Plant Physiol. **132**: 728–731. doi:10.1104/pp.102.019216.

Chan, C.W.M., Wohlbach, D.J., Rodesch, M.J., and Sussman, M.R. 2008. Transcriptional changes in response to growth of *Arabidopsis* in high external calcium. FEBS Lett. **582**: 967–976. doi:10.1016/j.febslet.2008.02.043. PMID:18307990.

Chin, K., Moeder, W., Mosher, S., Urquhart, W., and Yoshioka, K. 2008. The roles of the *Arabidopsis* cyclic nucleotide-gated ion channels, AtCNGC11 and 12 in abiotic and biotic stress responses. Annual Meeting of the Canadian Society of Plant Physiologists. 14–17 June 2008, Ottawa, Ont.

Christopher, D.A., Borsics, T., Yuen, C.Y., Ullmer, W., Andème-Ondzighi, C., Andres, M.A., Kang, B.H., and Staehelin, L.A.

2007. The cyclic nucleotide gated cation channel AtCNGC10 traffics from the ER via Golgi vesicles to the plasma membrane of *Arabidopsis* root and leaf cells. BMC Plant Biol. **7**: 48. doi:10.1186/1471-2229-7-48. PMID:17877833.

Chun, L., Kawakami, A., and Christopher, D.A. 2001. Phytochrome A mediates blue light and UV-A-dependent chloroplast gene transcription in green leaves. Plant Physiol. **125**: 1957–1966. doi:10.1104/pp.125.4.1957. PMID:11299375.

Clough, S.J., Fengler, K.A., Yu, I.-C., Lippok, B., Smith, R.K., Jr., and Bent, A.F. 2000. The *Arabidopsis dnd1* "defense, no death" gene encodes a mutated cyclic nucleotide-gated ion channel. Proc. Natl. Acad. Sci. U.S.A. **97**: 9323–9328. doi:10.1073/pnas.150005697. PMID:10900264.

Craven, K.B., and Zagotta, W.N. 2006. CNG and HCN channels: two peas, one pod. Annu. Rev. Physiol. **68**: 375–401. doi:10.1146/annurev.physiol.68.040104.134728. PMID:16460277.

Donaldson, L., Ludidi, N., Knight, M.R., Gehring, C., and Denby, K. 2004. Salt and osmotic stress cause rapid increases in *Arabidopsis thaliana* cGMP levels. FEBS Lett. **569**: 317–320. doi:10.1016/j.febslet.2004.06.016. PMID:15225654.

Durner, J., Wendehenne, D., and Klessig, D.F. 1998. Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP ribose. Proc. Natl. Acad. Sci. U.S.A. **95**: 10328–10333. doi:10.1073/pnas.95.17.10328. PMID:9707647.

Ehsan, H., Reichheld, J.P., Roef, L., Witters, E., Lardon, F., Bockstaele, D.V., Montagu, M.V., Inze, D., and Onckelen, H.V. 1998. Effect of indomethacin on cell cycle dependent cyclic AMP fluxes in tobacco BY-2 cells. FEBS Lett. **422**: 165–169. doi:10.1016/S0014-5793(97)01610-4. PMID:9489998.

Ehsan, H., Roef, L., Witters, E., Reichheld, J.P., Bockstaele, D.V., Inze, D., and Onckelen, H.V. 1999. Indomethacin-induced G1/S phase arrest of the plant cell cycle. FEBS Lett. **458**: 349–353. doi:10.1016/S0014-5793(99)01152-7. PMID:10570938.

Frietsch, S., Wang, Y.F., Sladek, C., Poulsen, L.R., Romanowsky, S.M., Schroeder, J.I., and Harper, J.F. 2007. A cyclic nucleotide-gated channel is essential for polarized tip growth of pollen. Proc. Natl. Acad. Sci. U.S.A. **104**: 14531–14536. doi:10.1073/pnas.0701781104. PMID:17726111.

Gobert, A., Park, G., Amtmann, A., Sanders, D., and Maathuis, F.J.M. 2006. *Arabidopsis thaliana* cyclic nucleotide gated channel 3 forms a non-selective ion transporter involved in germination and cation transport. J. Exp. Bot. **57**: 791–800. doi:10.1093/jxb/erj064. PMID:16449377.

Hatsugai, N., Kuroyanagi, M., Nishimura, M., and Hara-Nishimura, I. 2006. A cellular suicide strategy of plants: vacuole-mediated cell death. Apoptosis, **11**: 905–911. doi:10.1007/s10495-006-6601-1. PMID:16547592.

Hofmann, F., Dostmann, W., Keilbach, A., Landgraf, W., and Ruth, P. 1992. Structure and physiological role of cGMP-dependent protein kinase. Biochim. Biophys. Acta, **1135**: 51–60. doi:10.1016/0167-4889(92)90165-8. PMID:1317212.

Hua, B.G., Mercier, R.W., Leng, Q., and Berkowitz, G.A. 2003*a*. Plants do it differently. A new basis for potassium/sodium selectivity in the pore of an ion channel. Plant Physiol. **132**: 1353–1361. doi:10.1104/pp.103.020560. PMID:12857817.

Hua, B.G., Mercier, R.W., Zielinski, R.E., and Berkowitz, G.A. 2003*b*. Functional interaction of calmodulin with a plant cyclic nucleotide gated cation channel. Plant Physiol. Biochem. **41**: 945–954. doi:10.1016/j.plaphy.2003.07.006.

Jiang, J., Fan, L.W., and Wu, W.H. 2005. Evidences for involvement of endogenous cAMP in *Arabidopsis* defense responses to *Verticillium* toxins. Cell Res. **15**: 585–592. doi:10.1038/sj.cr.7290328. PMID:16117848.

Jurkowski, G.I., Smith, R.K., Yu, I.C., Ham, J.H., Sharma, S.B., Klessig, D.F., Fengler, K.A., and Bent, A.F. 2004. *Arabidopsis DND2*, a second cyclic nucleotide-gated ion channel gene for which mutation causes the "defense, no death" phenotype. Mol. Plant Microbe Interact. **17**: 511–520. doi:10.1094/MPMI.2004.17.5.511. PMID:15141955.

Kaplan, B., Sherman, T., and Fromm, H. 2007. Cyclic nucleotide-gated channels in plants. FEBS Lett. **581**: 2237–2246. doi:10.1016/j.febslet.2007.02.017. PMID:17321525.

Kaupp, U.B., and Seifert, R. 2002. Cyclic nucleotide-gated ion channels. Physiol. Rev. **82**: 769–824. PMID:12087135.

Ko, C.H., and Gaber, R.F. 1991. *TRK1* and *TRK2* encode structurally related $K^+$ transporters in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11**: 4266–4273. PMID:2072919.

Köhler, C., and Neuhaus, G. 2000. Characterization of calmodulin binding to cyclic nucleotide-gated ion channels from *Arabidopsis thaliana*. FEBS Lett. **471**: 133–136. doi:10.1016/S0014-5793(00)01383-1. PMID:10767408.

Köhler, C., Merkle, T., and Neuhaus, G. 1999. Characterization of a novel gene family of putative cyclic nucleotide- and calmodulin-regulated ion channels in *Arabidopsis thaliana*. Plant J. **18**: 97–104. doi:10.1046/j.1365-313X.1999.00422.x. PMID:10341447.

Köhler, C., Merkle, T., Roby, D., and Neuhaus, G. 2001. Developmentally regulated expression of cyclic nucleotide-gated ion channel from *Arabidopsis* indicates its involvement in programmed cell death. Planta, **213**: 327–332. doi:10.1007/s004250000510. PMID:11506354.

Kwezi, L., Meïer, S., Mungur, L., Ruzvidzo, O., Irving, H., and Gehring, C. 2007. The *Arabidopsis thaliana* brassinosteroid receptor (AtBRI1) contains a domain that functions as a guanylyl cyclase *in vitro*. PLoS One, **2**: e449. doi:10.1371/journal.pone.0000449. PMID:17520012.

Leng, Q., Mercier, R.W., Yao, W., and Berkowitz, G.A. 1999. Cloning and first functional characterization of a plant nucleotide-gated cation channel. Plant Physiol. **121**: 753–761. doi:10.1104/pp.121.3.753. PMID:10557223.

Leng, Q., Mercier, R.W., Hua, B.G., Fromm, H., and Berkowitz, G.A. 2002. Electrophysiological analysis of cloned cyclic nucleotide-gated ion channels. Plant Physiol. **128**: 400–410. doi:10.1104/pp.010832. PMID:11842144.

Li, X., Borsics, T., Harrington, H.M., and Christopher, D.A. 2005. *Arabidopsis* AtCNGC10 rescues potassium channel mutants of *E. coli*, yeast, and *Arabidopsis* and is regulated by calcium/calmodulin and cyclic GMP in *E. coli*. Funct. Plant Biol. **32**: 643–653. doi:10.1071/FP04233.

Ludidi, N., and Gehring, C. 2003. Identification of a novel protein with guanylyl cyclase activity in *Arabidopsis thaliana*. J. Biol. Chem. **278**: 6490–6494. doi:10.1074/jbc.M210983200. PMID:12482758.

Ma, W., Ali, R., and Berkowitz, G.A. 2006. Characterization of plant phenotypes associated with loss-of-function of AtCNGC1, a plant cyclic nucleotide gated cation channel. Plant Physiol. Biochem. **44**: 494–505. doi:10.1016/j.plaphy.2006.08.007. PMID:17027276.

Ma, W., Smigel, A., Tsai, Y.C., Braam, J., and Berkowitz, G.A. 2008. Innate immunity signaling: cytosolic $Ca^{2+}$ elevation is linked to downstream nitric oxide generation through the action of calmodulin or a calmodulin-like protein. Plant Physiol. **148**: 818–828. doi:10.1104/pp.108.125104. PMID:18689446.

Maathuis, F.J. 2006. cGMP modulates gene transcription and cation transport in *Arabidopsis* roots. Plant J. **45**: 700–711. doi:10.1111/j.1365-313X.2005.02616.x. PMID:16460505.

Maathuis, F.J.M., and Sanders, D. 2001. Sodium uptake in *Arabidopsis* roots is regulated by cyclic nucleotides. Plant Physiol. **127**: 1617–1625. doi:10.1104/pp.010502. PMID:11743106.

Martinez-Atienza, J., Van Ingelgem, C., Roef, L., and Maathuis, F.J.M. 2008. Plant cyclic nucleotide signaling. Plant Signal. Behav. **2**: 540–543.

Mäser, P., Thomine, S., Schroeder, J.I., Ward, J.M., Hirschi, K., Sze, H., Talke, I.N., Amtmann, A., Maathuis, F.J.M., Sanders, D., Harper, J.F., Tchieu, J., Gribskov, M., Persans, M.W., Salt, D.E., Kim, S.A., and Guerinot, M.L. 2001. Phylogenic relationship within cation transporter families of *Arabidopsis*. Plant Physiol. **126**: 1646–1667. doi:10.1104/pp.126.4.1646. PMID: 11500563.

Mercier, R.W., Rabinowitz, N.M., Ali, R., Gaxiola, R.A., and Berkowitz, G.A. 2004. Yeast hygromycin sensitivity as a functional assay of cyclic nucleotide gated cation channels. Plant Physiol. Biochem. **42**: 529–536. doi:10.1016/j.plaphy.2004.04.007. PMID:15246066.

Moncada, S., Marletta, M.A., Hibbs, J.B., and Higgs, E.A. (*Editors*). 1992. The biology of nitric oxide, Parts I–II. Portland Press, London, UK.

Moutinho, A., Hussey, P.J., Trewavas, A.J., and Malhó, R. 2001. cAMP acts as a second messenger in pollen tube growth and reorientation. Proc. Natl. Acad. Sci. U.S.A. **98**: 10481–10486. doi:10.1073/pnas.171104598. PMID:11517303.

Newton, R.P., and Smith, C.J. 2004. Cyclic nucleotides. Phytochemistry, **65**: 2423–2437. doi:10.1016/j.phytochem.2004.07.026. PMID:15381406.

Pagnussat, G.C., Lanteri, M.L., and Lamattina, L. 2003. Nitric oxide and cyclic GMP are messengers in the indole acetic acid-induced adventitious rooting process. Plant Physiol. **132**: 1241–1248. doi:10.1104/pp.103.022228. PMID:12857806.

Rall, T.W., Sutherland, E.W., and Berthet, J. 1957. The relation of epinephrine and glucagon to liver phosphorylase. J. Biol. Chem. **224**: 1987–1995.

Rehmann, H., Wittinghofer, A., and Bos, J.L. 2007. Capturing cyclic nucleotides in action: snapshots from crystallographic studies. Natl. Rev. **8**: 63–73. doi:10.1038/nrm2082.

Richards, H., Das, S., Smith, C.J., Pereira, L., Geisbrecht, A., Devitt, N.J., Games, D.E., van Geyschem, J., Gareth Brenton, A., and Newton, R.P. 2002. Cyclic nucleotide content of tobacco BY-2 cells. Phytochemistry, **61**: 531–537. doi:10.1016/S0031-9422(02)00266-2. PMID:12409019.

Rostoks, N., Schmierer, D., Mudie, S., Drader, T., Brueggeman, R., Caldwell, D.G., Waugh, R., and Kleinhofs, A. 2006. Barley necrotic locus nec1 encodes the cyclic nucleotide-gated ion channel 4 homologous to the Arabidopsis HLM1. Mol. Genet. Genomics, **275**: 159–168. doi:10.1007/s00438-005-0073-9. PMID:16341885.

Schuurink, R.C., Shartzer, S.F., Fath, A., and Jones, R.L. 1998. Characterization of a calmodulin-binding transporter from the plasma membrane of barley aleurone. Proc. Natl. Acad. Sci. U.S.A. **95**: 1944–1949. doi:10.1073/pnas.95.4.1944. PMID: 9465122.

Sunkar, R., Kaplan, B., Bouché, N., Arazi, T., Dolev, D., Talke, I.N., Maathuis, F.J.M., Sanders, D., Bouchez, D., and Fromm, H. 2000. Expression of a truncated tobacco NtCBP4 channel in transgenic plants and disruption of the homologous *Arabidopsis CNGC1* gene confer $Pb^{2+}$ tolerance. Plant J. **24**: 533–542. doi:10.1046/j.1365-313x.2000.00901.x. PMID:11115134.

Talke, I.N., Blaudez, D., Maathuis, F.J.M., and Sanders, D. 2003. CNGCs: prime targets of plant cyclic nucleotide signaling? Trends Plant Sci. **8**: 286–293. doi:10.1016/S1360-1385(03)00099-2. PMID:12818663.

Trudeau, M.C., and Zagotta, W.N. 2003. Calcium/calmodulin modulation of olfactory and rod cyclic nucleotide-gated ion channels. J. Biol. Chem. **278**: 18705–18708. doi:10.1074/jbc.R300001200.

Urquhart, W., Gunawardena, A.H.L.A.N., Moeder, W., Ali, R., Berkowitz, G.A., and Yoshioka, K. 2007. The chimeric cyclic nucleotide-gated ion channel ATCNGC11/12 constitutively induces programmed cell death in a $Ca^{2+}$ dependent manner. Plant Mol. Biol. **65**: 747–761. doi:10.1007/s11103-007-9239-7. PMID: 17885810.

Varnum, M.D., and Zagotta, W.N. 1997. Interdomain interactions underlying activation of cyclic nucleotide-gated channels. Science (Wash.), **278**: 110–113. doi:10.1126/science.278.5335.110. PMID:9311913.

Weston, E., Thorogood, K., Vinti, G., and Lopez-Juez, E. 2000. Light quantity controls leaf-cell and chloroplast development in *Arabidopsis thaliana* wild type and blue-light-perception mutants. Planta, **211**: 807–815. doi:10.1007/s004250000392. PMID:11144265.

White, P.J., Bowen, H.C., Demidchik, V., Nichols, C., and Davies, J.M. 2002. Genes for calcium-permeable channels in the plasma membrane of plant root cells. Biochim. Biophys. Acta, **1564**: 299–309. doi:10.1016/S0005-2736(02)00509-6. PMID:12175911.

Wissinger, B., Gamer, D., and Jagle, H. 2001. CNGA3 mutations in hereditary cone photoreceptor disorders. Am. J. Hum. Genet. **69**: 722–733. doi:10.1086/323613. PMID:11536077.

Yoshioka, K., Kachroo, P., Tsui, F., Sharma, S.B., Shah, J., and Klessig, D.F. 2001. Environmentally sensitive, SA-dependent defense responses in the *cpr22* mutant of *Arabidopsis*. Plant J. **26**: 447–459. doi:10.1046/j.1365-313X.2001.2641039.x. PMID: 11439131.

Yoshioka, K., Moeder, W., Kang, H.G., Kachroo, P., Masmoudi, K., Berkowitz, G., and Klessig, D.F. 2006. The chimeric *Arabidopsis* cyclic nucleotide-gated ion channel 11/12 activates multiple pathogen resistance responses. Plant Cell, **18**: 747–763. doi:10.1105/tpc.105.038786. PMID:16461580.

Yu, I.C., Parker, J., and Bent, A.F. 1998. Gene-for-gene disease resistance without the hypersensitive response in *Arabidopsis dnd1* mutant. Proc. Natl. Acad. Sci. U.S.A. **95**: 7819–7824. doi:10.1073/pnas.95.13.7819. PMID:9636234.

Zagotta, W.N., and Siegelbaum, S.A. 1996. Structure and function of cyclic nucleotide-gated channels. Annu. Rev. Neurosci. **19**: 235–263. doi:10.1146/annurev.ne.19.030196.001315. PMID: 8833443.

Zagotta, W.N., Olivier, N.B., Black, K.D., Young, E.C., Rolson, R., and Gouaux, E. 2003. Structural basis for modulation and agonist specificity of HCN pacemaker channel. Nature (London), **425**: 200–205. doi:10.1038/nature01922. PMID:12968185.

Zheng, J., and Zagotta, W.N. 2004. Stoichiometry and assembly of olfactory cyclic nucleotide-gated channels. Neuron, **42**: 411–421. doi:10.1016/S0896-6273(04)00253-3. PMID:15134638.

MINIREVIEW / MINISYNTHÈSE

# Auxin-transport-dependent leaf vein formation[1]

**Tyler J. Donner and Enrico Scarpella**

**Abstract:** The formation of vein patterns in leaves has captivated biologists, mathematicians, and philosophers. In leaf development, files of vein-forming procambial cells emerge from within a seemingly homogeneous subepidermal tissue through the selection of anatomically inconspicuous preprocambial cells. Although the molecular details underlying the orderly formation of veins in the leaf remain elusive, gradually restricted transport paths of the plant signaling molecule auxin have long been implicated in defining sites of vein differentiation. Several recent advances converge to more precisely define the role of auxin flow at successive stages of vascular development. The picture that emerges is that of vein formation as a self-organizing, reiterative, auxin-transport-dependent process.

*Key words: Arabidopsis*, leaf development, polar auxin transport, procambium, vascular patterning.

**Résumé :** La formation des patrons des nervures foliaires a captivé les biologistes, les mathématiciens et les philosophes. Au cours du développement foliaire, des files de cellules procambiales forment des veines émergentes au sein de ce qui semble un tissus sub épidermique homogène, via la sélection de cellules procambiales anatomiquement invisibles. Bien que les détails moléculaires sous-jacents à la formation ordonnée des nervures dans la feuille demeurent insaisissables, on a depuis longtemps impliqué le transport graduellement restreint de la molécule d'auxine responsable de signaux végétaux, dans la définition des sites de différenciation des nervures. Plusieurs percées récentes convergent vers une définition plus précise du rôle du flux d'auxine à différents stades du développement vasculaire. Le tableau qui émerge est celui de la formation des nervures comme un processus auto organisé, réitératif et dépendant de l'auxine.

*Mots-clés : Arabidopsis*, développement foliaire, transport polaire de l'auxine, procambium, formation du patron vasculaire.

[Traduit par la Rédaction]

## Introduction

The vascular system of plants consists of a network of cell files (vascular strands) that extends through all organs (Esau 1965). In dicot leaves, these vascular strands, or veins, are arranged in a ramified pattern that largely reflects the shape of the leaf (Nelson and Dengler 1997; Dengler and Kang 2001) (Fig. 1A). Lateral veins branch from a conspicuous central vein (midvein) that is continuous with the stem vasculature. In many species, lateral veins extend along the leaf edge to form marginal veins, which connect to distal veins to form prominent closed loops. Finally, a series of higher-order veins branch from the midvein and the loops, and can either terminate in the lamina (free-ending veins) or join two veins (connected veins).

Received 31 October 2008. Published on the NRC Research Press Web site at botany.nrc.ca on 10 July 2009.

**T.J. Donner and E. Scarpella.**[2] Department of Biological Sciences, University of Alberta, CW-405 Biological Sciences Building, Edmonton AB T6G 2E9, Canada.

[1]This paper is one of a selection published in a Special Issue comprising papers presented at the 50th Annual Meeting of the Canadian Society of Plant Physiologists (CSPP) held at the University of Ottawa, Ontario, in June 2008.

[2]Corresponding author (e-mail: enrico.scarpella@ualberta.ca).

All vascular cell types mature from procambial cells: narrow, cytoplasm-dense cells, characteristically arranged in continuous lines (Esau 1943). In the leaf, procambial strands differentiate from files of isodiametric "preprocambial" cells, which are selected from the anatomically homogeneous subepidermal tissue of the leaf primordium, the ground meristem (Foster 1952; Pray 1955; Mattsson et al. 2003; Kang and Dengler 2004; Scarpella et al. 2004) (Fig. 1B). The mechanism by which ground meristem cells are specified to procambial cell fate is unknown, but an instrumental role for the polar transport and resulting distribution patterns of the plant signaling molecule auxin in this process has increasingly gained support (Sachs 1981, 1989; Mattsson et al. 1999; Sieburth 1999; Mattsson et al. 2003; Scarpella et al. 2006; Wenzel et al. 2007).

A variety of substances have been reported to promote vascular differentiation (Fukuda 2004); however, the role of auxin in this process is unique. Auxin is the only molecule that not only triggers vascular cell differentiation, but also induces vascular strand formation, a response with distinctive and reproducible properties (Sachs 1981; Berleth et al. 2000) (Fig. 1C). Application of auxin to plant tissues results in the formation of new vascular strands that connect the auxin source with pre-existing vasculature. The auxin-induced vascular differentiation response is (*i*) local, as vascular strand formation is initiated at the specific site of

 doi:10.1139/B09-002 

auxin application; (*ii*) polar, as vascular strand formation progresses from the auxin source towards the basal side of the plant; (*iii*) continuous, as it generates uninterrupted files of vascular cells; (*iv*) constrained in the planes perpendicular to the main axis of the vascular differentiation response, as it originates multiple, slender bundles of vascular cell files; and (*v*) dependent on auxin flow, as it requires efficiently transported auxins and is obstructed in the presence of auxin transport inhibitors. The capability of a simple signal to trigger a complex and oriented cellular response such as that of vascular strand formation suggests that the underlying mechanism recruits directional cues already present in the organism. Because in the plant auxin is synthesized predominantly in young apical regions, such as leaf primordia and floral buds, and transported basally (Michniewicz et al. 2007), the source of asymmetric information co-opted by the auxin-induced vascular differentiation response probably coincides with the polar flow of auxin itself. This brief review summarizes and integrates the findings of a recent group of studies that together have generated a conceptual framework for experimentally exploring functions of auxin transport in leaf vein formation at the cellular and molecular levels. For other aspects of leaf vascular development, the reader is referred to a series of thoughtful reviews (e.g., Nelson and Dengler 1997; Berleth et al. 2000; Dengler and Kang 2001; Sieburth and Deyholos 2006; Rolland-Lagan 2008).

**Leaf initiation and vein positioning**

Paths of polar flow of the auxin signal seems to be accurately visualized through the subcellular localization of auxin exporters of the PIN family (Petrasek et al. 2006; Wisniewska et al. 2006), and asymmetric distribution of PIN1 at the plasma membrane of epidermal cells at the shoot apex suggests that auxin transport routes converge towards sites of leaf primordium formation (Benkova et al. 2003; Reinhardt et al. 2003; Heisler et al. 2005) (Fig. 1D). "Convergence points" of PIN1 polarity in the epidermis identifying locations of primordium initiation become associated with subsequently emerging subepidermal domains of PIN1 expression that will define the position of midvein formation. Similarly, lateral outgrowth of the leaf primordium and positioning of lateral vein-forming PIN1 subepidermal domains seem to be anchored to one another through epidermal PIN1 convergence points at the primordium margin (Hay et al. 2006; Scarpella et al. 2006; Wenzel et al. 2007) (Fig. 1E).

General reduction in cell division during leaf development results in smaller leaves with fewer lateral veins, while excessive cell proliferation throughout the entire leaf gives rise to larger leaves with supernumerary loops (Kang et al. 2007). In both cases, however, spacing of convergence point-associated lateral veins seems to be preserved, suggesting that global cell division activity merely produces the substrate on which the vein patterning mechanism acts. Such flexibility in the vein formation process, also observed in a variety of other experimental conditions (e.g., Sachs 1989; Mattsson et al. 1999; Sieburth 1999; Scarpella et al. 2006), is difficult to reconcile with a developmental program in which the position of veins is rigidly predetermined, suggesting that the vein patterning mechanism is a self-organizing and propagating process.

Excess auxin resulting from local auxin application or systemic auxin transport inhibition reduces spacing of epidermal convergence points in the leaf and leads to the surplus formation of closely spaced loops, similar to vein arrangements observed in *pin1* mutant leaves (Mattsson et al. 1999; Sieburth 1999; Scarpella et al. 2006). Consistent with a functional similarity between midvein and lateral vein positioning in the leaf and primordium formation at the shoot apex, auxin overload (through direct auxin application or auxin transport inhibition) also reduces separation between successively initiated leaf primordia (Okada et al. 1991; Bennett et al. 1995; Reinhardt et al. 2000). These observations suggest that positioning of convergence points is strictly dependent on auxin supply and transport, but a contribution of auxin uptake in stabilizing this process has more recently been uncovered. Simultaneous mutation in multiple members of the AUX1/LAX family of auxin importers results in reduced frequency and altered distribution of convergence points at the shoot apex, which are associated with spatially disorganized initiation of leaf primordia and which occur in the absence of apparent alterations in shoot apex size and structure (Bainbridge et al. 2008). However, these defects are only observed under specific environmental conditions and at specific developmental stages, and it remains to be addressed whether a similar function in convergence point patterning can be attributed to AUX1/LAX proteins in leaf primordia.

**Preprocambial cell selection**

Expression profiling identifies *PIN1* as the most relevant member of the *PIN* gene family in leaf vein formation (Scarpella et al. 2006). During normal and experimentally altered leaf vein formation, PIN1 expression in subepidermal cells precedes and converges towards sites of preprocambial strand formation and, at the PIN1 expression level, all veins appear to be generated through two basic ontogenies (Scarpella et al. 2006; Wenzel et al. 2007) (Fig. 1D and 1E). The midvein and lateral veins originate from subepidermal PIN1 domains associated with convergence points of PIN1 polarity in the epidermis, while higher-order veins emerge from PIN1 domains initiated within the expanding lamina. These internal domains are initially free-ending, but can become connected upon proximity to other PIN1 domains. Interestingly, individual loops are composed of a lateral PIN1 domain, which is initiated at an epidermal convergence point, and a marginal domain, which is ontogenically equivalent to a connected higher-order PIN1 domain (Fig. 1E and 1F(*a*)). In mature leaves, the composite origin of the first and second loop pairs is obscured by the smooth amalgamation of lateral and marginal veins, but this origin can be recognized in third and subsequent loop pairs (Kang and Dengler 2004; Scarpella et al. 2006) (Fig. 1A).

Heightened auxin levels, occurring naturally in association with hydathodes or experimentally as a consequence of either direct auxin application or auxin transport inhibition, lead to the expansion of PIN1 expression domains (Aloni et al. 2003; Mattsson et al. 2003; Hay et al. 2006; Scarpella et al. 2006; Wenzel et al. 2007) (Fig. 1F(*a*)). Broad PIN1 domains eventually taper to a few cell files that predict vein position, and this narrowing process is dependent on auxin transport. Subcellular PIN1 localization indicates that PIN1

**Fig. 1.** Control of leaf vein formation by polar auxin transport. (A) Schematics of a simplified mature leaf illustrating midvein (M), first, second, and third loops (L1, L2, and L3, respectively), each derived from corresponding lateral (LV) and marginal (MV) veins, free-ending (FV) and connected (CV) higher-order veins, hydathodes (H), and middle-to-margin positions (decreasing green gradient) as used in the text. (B) State transitions in leaf subepidermal cell differentiation. Available evidence suggests that the vein patterning process is limited to ground meristem cells (white), while subepidermal cells that have begun to acquire mesophyll characteristics are incapable of responding to vein-inducing signals (Scarpella et al. 2004; Scarpella et al. 2006; Kang et al. 2007; Wenzel et al. 2007). The transition from ground meristem to differentiated mesophyll occurs through a "premesophyll" cell state that seems to be formally equivalent to the preprocambial state in vascular differentiation (Sawchuk et al. 2008). Expression of preprocambial (cyan) and premesophyll (yellow) markers identifies two mutually exclusive and seemingly irreversible cell states, one leading to procambium (purple) and the other to mature mesophyll (green) formation (Scarpella et al. 2004; Sawchuk et al. 2008). However, the degree of stability of the premesophyll state, the mutual exclusivity or competition of its acquisition with selection of preprocambial cells, and its responsiveness to vein-inducing signals still remain open questions. (C) Vascular strand formation in response to local auxin application. A source of auxin (brown) locally applied to a hypocotyl segment triggers the formation of vascular strands (orange) that connect the auxin source to an existing vascular strand (grey). While the precise path of vascular strand formation is not reproducible, the differentiation process always proceeds towards the basal pole of the plant, is restricted to narrow bundles of cell files, leads to continuity with existing vasculature, and requires functional auxin transport, suggesting integrating mechanisms acting along the length of the strand. The drawing summarizes findings reviewed in (Sachs 1981). (D and E) Routes of auxin transport (black arrows) and convergence points of auxin flow (brown) in leaf primordium initiation (D) and vein formation (E) as inferred from subcellular localization of PIN1. The midvein and lateral veins (orange) are associated with epidermal convergence of auxin transport, while marginal veins that form the upper part of each vein loop represent the extension of higher-order veins (dark yellow) that otherwise end freely in the lamina. (F) Stage-specific dynamics of leaf vein patterning and their dependency on auxin levels and transport as exemplified for loop formation, but in general equally applicable to all veins. (*a*) PIN1-labeled auxin transport paths corresponding to preprocambial cell selection zones (pink). Note how loops are composed of a lateral PIN1 expression domain (LD) and an initially free-ending marginal PIN1 expression domain (MD). Further, note slightly expanded PIN1 expression domains in a fraction of hydathode-associated third loops during normal development, broad PIN1 domains on the side of local auxin application (arrowhead) and nearly ubiquitous PIN1 expression upon systemic auxin transport inhibition. (*b*) Directions of *Athb8*/J1721-defined preprocambial strand formation (cyan arrows). Note middle-to-margin progression of preprocambial strand formation during normal loop development. Further, note margin-to-middle preprocambial strand extension in a fraction of third loops during normal development and in all loops forming on the side of auxin application. Finally, note co-existence of middle-to-margin and margin-to-middle polarities of preprocambial strand extension during the formation of individual loops in response to auxin transport inhibition. (*c*) Gradual appearance of ET1335/Q0990-marked procambial cell identity acquisition (light to dark purple). Note simultaneous differentiation of lateral and marginal procambial strands in normal loop development. Further, note successive formation of lateral and marginal procambial strands in a fraction of third loops during normal development and in all loops formed on the side of auxin application and under conditions of reduced auxin transport. Arrows temporally connect successive stages of vein formation. See text for additional details.

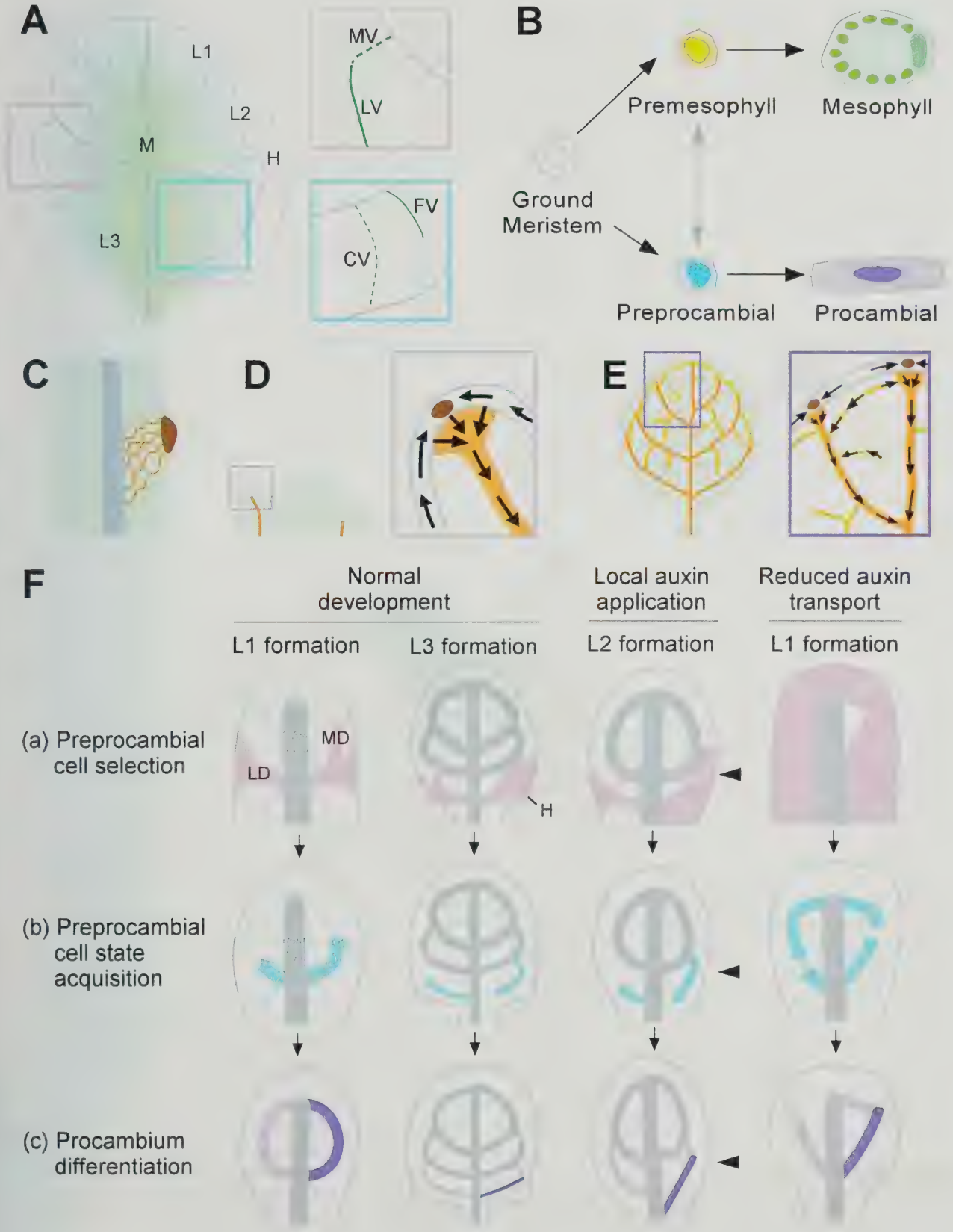
polarity may not be uniformly directed towards pre-existing veins across wide PIN1 domains, but it is usually so along each domain's midline. Within narrow PIN1 domains, subcellular localization indicates auxin transport towards pre-existing veins: in free-ending domains, a single polarity exists, while in connected domains, two opposite polarities are connected by a bipolar cell (Scarpella et al. 2006; Wenzel et al. 2007).

These observations are consistent with the notion that vasculature is formed along core areas of gradually restricted domains of elevated auxin transport (Mitchison 1980; Sachs 1989; Rolland-Lagan and Prusinkiewicz 2005). However, traditional models of auxin transport, which rely on high levels of auxin exporter expression in cells specialized for auxin transport, simulate auxin depletion from developing veins. These predictions are difficult to reconcile with experimental evidence suggesting that veins develop along paths of maximum auxin levels (Mattsson et al. 2003; Scarpella et al. 2004, 2006), but supplementing conventional auxin export-based models with carrier-mediated auxin uptake seems to be sufficient to maintain high auxin concentrations within developing veins (Kramer 2004). While auxin still remains "invisible" at the cellular level, this projection can now be experimentally evaluated as multiple mutant combinations of members of the AUX1/LAX family of auxin importers have recently become available (Bainbridge et al. 2008).

## Preprocambial cell state acquisition

During the formation of all veins and under all tested experimental conditions, expression of preprocambial cell state markers such as *Athb8* and J1721 is initiated next to pre-existing vasculature and extends progressively away from its point of origin (Kang and Dengler 2004; Scarpella et al. 2004; Sawchuk et al. 2007), suggesting that all veins arise as free-ending preprocambial branches (Fig. 1F(*b*)). Furthermore, connected veins form by fusion of initially free-ending preprocambial strands with other free-ending or connected strands, while free-ending veins result from termination of the extension of preprocambial expression domains (Scarpella et al. 2004; Sawchuk et al. 2007) (Fig. 1F(*b*)).

Under all conditions, preprocambial strands extend progressively, although the specific direction of this progression varies. Preprocambial strands in the first and second loop pairs invariably extend from central to marginal regions of the developing leaf, while third loop preprocambial strands can form in the opposite direction (i.e., marginal to central) (Sawchuk et al. 2007) (Fig. 1F(*b*)). Unlike the first and second loop pairs, third loop pairs are associated with expanded PIN1 subepidermal domains and conspicuous auxin response maxima at the primordium margin (Aloni et al. 2003; Mattsson et al. 2003; Hay et al. 2006; Scarpella et al. 2006; Wenzel et al. 2007) (Fig. 1F(*b*)). This suggests that preprocambial strands are initiated at a critical auxin level, which for third and subsequent loop pairs could be attained at the margin


A
L1
L2
M
H
L3
MV
LV
FV
CV
B
Premesophyll
Mesophyll
Ground Meristem
Preprocambial
Procambial
C
D
E
F
Normal development
Local auxin application
Reduced auxin transport
L1 formation
L3 formation
L2 formation
L1 formation
(a) Preprocambial cell selection
LD
MD
H
(b) Preprocambial cell state acquisition
(c) Procambium differentiation

of the primordium, possibly because of localized auxin synthesis (Cheng et al. 2006). In contrast, auxin levels critical for initiation of preprocambial strands of the first two loop pairs would be achieved at the centre of the primordium, in proximity of the midvein, presumably where auxin produced throughout the lamina converges. This interpretation is further supported by the observation that direct auxin application at the primordium margin results solely in margin-to-middle polarity of preprocambial strand formation (Sawchuk et al. 2007) (Fig. 1F(*b*)). Moreover, when auxin levels are uniformly increased throughout the primordium because of reduction in auxin flow, individual loops can even be formed by fusion of two preprocambial strands extending with opposed polarities (Sawchuk et al. 2007) (Fig. 1F(*b*)).

**Procambium differentiation**

Expression of procambial differentiation markers, including ET1335 and Q0990, suggests that procambium distinctive features appear simultaneously along entire strands (Mattsson et al. 1999; Scarpella et al. 2004; Sawchuk et al. 2007) (Fig. 1F(*c*)). Preprocambial cells within an individual strand acquire the stereotypically narrow shape of procambium through coordinated elongation, rather than through synchronized cell division parallel to the axis of the preprocambial strand (Donnelly et al. 1999; Kang and Dengler 2002; Sawchuk et al. 2007).

While procambium differentiates simultaneously along the complete loop in the first two loop pairs, lateral and marginal procambial strands can appear successively in third loops (Sawchuk et al. 2007) (Fig. 1F(*c*)). As the formation of third loop pairs is associated with increased auxin levels at the hydathode (Aloni et al. 2003; Mattsson et al. 2003; Cheng et al. 2006; Hay et al. 2006; Scarpella et al. 2006), excess auxin seems to prevent simultaneous procambium differentiation along entire loops. This hypothesis is also supported by the observation that lateral and marginal procambial strands appear separately in all loop-like veins formed in response to auxin application (Sawchuk et al. 2007) (Fig. 1F(*c*)). Furthermore, when auxin levels are raised in the primordium through auxin transport inhibition, differentiation of lateral and marginal strands occurs in temporally distinct steps for all loops, including the first two loop pairs, which typically undergo simultaneous differentiation (Sawchuk et al. 2007) (Fig. 1F(*c*)). Augmented auxin levels could lead to deviations in simultaneity of procambial loop differentiation by delaying the transition of incipient lateral veins from less efficient to more efficient sinks. This, in turn, would defer the formation of marginal veins connecting lateral veins to pre-existing vasculature, which is similar to what is observed in other organs (Sachs 1968). The simultaneous differentiation of lateral and marginal procambial strands in first and second loop pairs observed under normal conditions could thus simply reflect efficient auxin flow and (or) inconspicuous auxin synthesis in early primordium development.

**Termination of vein formation**

Termination of vein formation could, in principle, occur at any developmental stage; however, available evidence suggests that, although formally possible, termination is unlikely to occur at the procambial stage. In fact, all the mutants isolated to date with fragmented vein patterns show defects in continuity of *Athb8* preprocambial expression domains in the presence of initially uninterrupted files of PIN1-selected preprocambial cells (Carland et al. 1999; Deyholos et al. 2000; Koizumi et al. 2000; Carland and Nelson 2004; Scarpella et al. 2006). Furthermore, mutants with fewer procambial strands, a higher proportion of which end freely, display comparable reduction in complexity and connectivity of *Athb8* expression territories (Candela et al. 1999; Alonso-Peral et al. 2006; Cnops et al. 2006; Petricka and Nelson 2007). These observations suggest that termination of vein formation must have occurred during preprocambial cell state acquisition. Termination of preprocambial strand formation has, in turn, been suggested to be a product of exhausted signaling within the developing veins (Carland et al. 1999; Steynen and Schultz 2003; Carland and Nelson 2004; Motose et al. 2004) and (or) consumptive differentiation of the remaining ground meristem population into the alternative subepidermal tissue of the mature leaf, the mesophyll (Scarpella et al. 2004; Kang et al. 2007). Whether these different mechanisms are elements of the same pathway will have to await the identification of more molecular components.

**Prospects**

How vein patterns can be generated has been subject to discussion, and although pieces of the puzzle have started to emerge, many questions remain unanswered. Placement of leaf primordia at the shoot apex and location of midvein and lateral veins within developing leaves is strictly associated with sites of converging auxin flow in epidermal cells. What is the molecular mechanism underlying generation and positioning of convergence points of auxin transport? The existence of bipolar cells provides a possible explanation to reconcile polarity of auxin flow with closed vein networks, in which most veins are connected to others at both ends with no obvious polarity. Are bipolar cells located at fixed positions within individual connected veins or does their position along the course of each vein change over time? And do those cells represent local sources of auxin? Different stages of vein formation display strikingly different dynamics (Fig. 1F(*a–c*)). How are developmental dynamics of one stage translated into those characteristic of the subsequent stage? Expression of preprocambial and premesophyll markers identify two non-overlapping cell states (Sawchuk et al. 2008) (Fig. 1B). How are developmental decisions made in vein-forming cells coordinated with decisions made in non-vascular tissues? While the answers to these and other questions still elude us, the recent work reviewed here has originated a scaffold of hypotheses and derived predictions that can be directly tested in the near future.

## Acknowledgements

This work was supported by a Discovery Grant of the Natural Sciences and Engineering Research Council of Canada, by an Alberta Ingenuity New Faculty Grant, and by the Canada Research Chairs Program; T.J.D. was supported by an Alberta Ingenuity Student Scholarship.

## References


Aloni, R., Schwalm, K., Langhans, M., and Ullrich, C.I. 2003. Gradual shifts in sites of free-auxin production during leaf-primordium development and their role in vascular differentiation and leaf morphogenesis in *Arabidopsis*. Planta, **216**(5): 841–853. PMID:12624772.

Alonso-Peral, M.M., Candela, H., del Pozo, J.C., Martinez-Laborda, A., Ponce, M.R., and Micol, J.L. 2006. The *HVE/CAND1* gene is required for the early patterning of leaf venation in *Arabidopsis*. Development, **133**(19): 3755–3766. doi:10.1242/dev.02554. PMID: 16943276.

Bainbridge, K., Guyomarc'h, S., Bayer, E., Swarup, R., Bennett, M., Mandel, T., and Kuhlemeier, C. 2008. Auxin influx carriers stabilize phyllotactic patterning. Genes Dev. **22**(6): 810–823. doi:10.1101/gad.462608. PMID:18347099.

Benkova, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertova, D., Jurgens, G., and Friml, J. 2003. Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell, **115**(5): 591–602. doi:10.1016/S0092-8674(03)00924-3. PMID:14651850.

Bennett, S.R.M., Alvarez, J., Bossinger, G., and Smyth, D.R. 1995. Morphogenesis in *pinoid* mutants of *Arabidopsis thaliana*. Plant J. **8**(4): 505–520. doi:10.1046/j.1365-313X.1995.8040505.x.

Berleth, T., Mattsson, J., and Hardtke, C.S. 2000. Vascular continuity and auxin signals. Trends Plant Sci. **5**(9): 387–393. doi:10.1016/S1360-1385(00)01725-8. PMID:10973094.

Candela, H., Martinez-Laborda, A., and Micol, J.L. 1999. Venation pattern formation in *Arabidopsis thaliana* vegetative leaves. Dev. Biol. **205**(1): 205–216. doi:10.1006/dbio.1998.9111. PMID:9882508.

Carland, F.M., and Nelson, T. 2004. *COTYLEDON VASCULAR PATTERN2*-mediated inositol (1,4,5) triphosphate signal transduction is essential for closed venation patterns of *Arabidopsis* foliar organs. Plant Cell, **16**(5): 1263–1275. doi:10.1105/tpc.021030. PMID:15100402.

Carland, F.M., Berg, B.L., FitzGerald, J.N., Jinamornphongs, S., Nelson, T., and Keith, B. 1999. Genetic regulation of vascular tissue patterning in *Arabidopsis*. Plant Cell, **11**(11): 2123–2137. doi:10.1105/tpc.11.11.2123. PMID:10559439.

Cheng, Y., Dai, X., and Zhao, Y. 2006. Auxin biosynthesis by the YUCCA flavin monooxygenases controls the formation of floral organs and vascular tissues in *Arabidopsis*. Genes Dev. **20**(13): 1790–1799. doi:10.1101/gad.1415106. PMID:16818609.

Cnops, G., Neyt, P., Raes, J., Petrarulo, M., Nelissen, H., Malenica, N., Luschnig, C., Tietz, O., Ditengou, F., Palme, K., Azmi, A., Prinsen, E., and Van Lijsebettens, M. 2006. The *TORNADO1* and *TORNADO2* genes function in several patterning processes during early leaf development in *Arabidopsis thaliana*. Plant Cell, **18**(4): 852–866. doi:10.1105/tpc.105.040568. PMID:16531491.

Dengler, N., and Kang, J. 2001. Vascular patterning and leaf shape. Curr. Opin. Plant Biol. **4**(1): 50–56. doi:10.1016/S1369-5266(00)00135-7. PMID:11163168.

Deyholos, M.K., Cordner, G., Beebe, D., and Sieburth, L.E. 2000. The *SCARFACE* gene is required for cotyledon and leaf vein patterning. Development, **127**(15): 3205–3213. PMID:10887077.

Donnelly, P.M., Bonetta, D., Tsukaya, H., Dengler, R.E., and Dengler, N.G. 1999. Cell cycling and cell enlargement in developing leaves of *Arabidopsis*. Dev. Biol. **215**(2): 407–419. doi:10.1006/dbio.1999.9443. PMID:10545247.

Esau, K. 1943. Origin and development of primary vascular tissues in plants. Bot. Rev. **9**: 125–206. doi:10.1007/BF02872466.

Esau, K. 1965. Plant anatomy. John Wiley, New York; London, UK.

Foster, A.S. 1952. Foliar venation in angiosperms from an ontogenetic standpoint. Am. J. Bot. **39**(10): 752–766. doi:10.2307/2438624.

Fukuda, H. 2004. Signals that control plant vascular cell differentiation. Nat. Rev. Mol. Cell Biol. **5**(5): 379–391. doi:10.1038/nrm1364. PMID:15122351.

Hay, A., Barkoulas, M., and Tsiantis, M. 2006. *ASYMMETRIC LEAVES1* and auxin activities converge to repress *BREVIPEDICELLUS* expression and promote leaf development in *Arabidopsis*. Development, **133**(20): 3955–3961. doi:10.1242/dev.02545. PMID:16971475.

Heisler, M.G., Ohno, C., Das, P., Sieber, P., Reddy, G.V., Long, J.A., and Meyerowitz, E.M. 2005. Patterns of auxin transport and gene expression during primordium development revealed by live imaging of the *Arabidopsis* inflorescence meristem. Curr. Biol. **15**(21): 1899–1911. doi:10.1016/j.cub.2005.09.052. PMID:16271866.

Kang, J., and Dengler, N. 2002. Cell cycling frequency and expression of the homeobox gene *ATHB-8* during leaf vein development in *Arabidopsis*. Planta, **216**(2): 212–219. doi:10.1007/s00425-002-0847-9. PMID:12447534.

Kang, J., and Dengler, N. 2004. Vein pattern development in adult leaves of *Arabidopsis thaliana*. Int. J. Plant Sci. **165**(2): 231–242. doi:10.1086/382794.

Kang, J., Mizukami, Y., Wang, H., Fowke, L., and Dengler, N.G. 2007. Modification of cell proliferation patterns alters leaf vein architecture in *Arabidopsis thaliana*. Planta, **226**(5): 1207–1218. doi:10.1007/s00425-007-0567-2. PMID:17569988.

Koizumi, K., Sugiyama, M., and Fukuda, H. 2000. A series of novel mutants of *Arabidopsis thaliana* that are defective in the formation of continuous vascular network: calling the auxin signal flow canalization hypothesis into question. Development, **127**(15): 3197–3204. PMID:10887076.

Kramer, E.M. 2004. PIN and AUX/LAX proteins: their role in auxin accumulation. Trends Plant Sci. **9**(12): 578–582. doi:10.1016/j.tplants.2004.10.010. PMID:15564124.

Mattsson, J., Sung, Z.R., and Berleth, T. 1999. Responses of plant vascular systems to auxin transport inhibition. Development, **126**(13): 2979–2991. PMID:10357941.

Mattsson, J., Ckurshumova, W., and Berleth, T. 2003. Auxin signaling in *Arabidopsis* leaf vascular development. Plant Physiol. **131**(3): 1327–1339. doi:10.1104/pp.013623. PMID:12644682.

Michniewicz, M., Brewer, P.B., and Friml, J. 2007. Polar auxin transport and asymmetric auxin distribution. *In* The *Arabidopsis* Book. American Society of Plant Biologists, Rockville, Md. [Online, available from www.aspb.org/publications/arabidopsis]. doi:10.1199/tab.0108.

Mitchison, G.J. 1980. Model for vein formation in higher-plants. Proc. R. Soc. Lond. B. Biol. Sci. **207**(1166): 79–109.

Motose, H., Sugiyama, M., and Fukuda, H. 2004. A proteoglycan mediates inductive interaction during plant vascular development. Nature (London), **429**(6994): 873–878. doi:10.1038/nature02613. PMID:15215864.

Nelson, T., and Dengler, N. 1997. Leaf vascular pattern formation. Plant Cell, **9**(7): 1121–1135. doi:10.1105/tpc.9.7.1121. PMID: 12237378.

Okada, K., Ueda, J., Komaki, M.K., Bell, C.J., and Shimura, Y. 1991. Requirement of the auxin polar transport system in early stages of *Arabidopsis* floral bud formation. Plant Cell, **3**(7): 677–684. doi:10.1105/tpc.3.7.677. PMID:12324609.

Petrasek, J., Mravec, J., Bouchard, R., Blakeslee, J.J., Abas, M., Seifertova, D., Wisniewska, J., Tadele, Z., Kubes, M., Covanova, M., Dhonukshe, P., Skupa, P., Benkova, E., Perry, L., Krecek, P., Lee, O.R., Fink, G.R., Geisler, M., Murphy, A.S., Luschnig, C., Zazimalova, E., and Friml, J. 2006. PIN proteins perform a rate-limiting function in cellular auxin efflux. Science (Wash.), **312**(5775): 914–918. doi:10.1126/science.1123542. PMID:16601150.

Petricka, J.J., and Nelson, T.M. 2007. *Arabidopsis* nucleolin affects plant development and patterning. Plant Physiol. **144**(1): 173–186. doi:10.1104/pp.106.093575. PMID:17369435.

Pray, T.R. 1955. Foliar venation of Angiosperms. IV. Histogenesis of the venation of Hosta. Am. J. Bot. **42**(8): 698–706. doi:10.2307/2438644.

Reinhardt, D., Mandel, T., and Kuhlemeier, C. 2000. Auxin regulates the initiation and radial position of plant lateral organs. Plant Cell, **12**(4): 507–518. doi:10.1105/tpc.12.4.507. PMID:10760240.

Reinhardt, D., Pesce, E.R., Stieger, P., Mandel, T., Baltensperger, K., Bennett, M., Traas, J., Friml, J., and Kuhlemeier, C. 2003. Regulation of phyllotaxis by polar auxin transport. Nature (London), **426**(6964): 255–260. doi:10.1038/nature02081. PMID:14628043.

Rolland-Lagan, A.G. 2008. Vein patterning in growing leaves: axes and polarities. Curr. Opin. Genet. Dev. **18**(4): 348–353. doi:10.1016/j.gde.2008.05.002. PMID:18606536.

Rolland-Lagan, A.G., and Prusinkiewicz, P. 2005. Reviewing models of auxin canalization in the context of leaf vein pattern formation in *Arabidopsis*. Plant J. **44**(5): 854–865. doi:10.1111/j.1365-313X.2005.02581.x. PMID:16297075.

Sachs, T. 1968. On determination of pattern of vascular tissues in peas. Ann. Bot. (Lond.), **32**(128): 781–790.

Sachs, T. 1981. The control of the patterned differentiation of vascular tissues. Adv. Bot. Res. **9**: 151–262. doi:10.1016/S0065-2296(08)60351-1.

Sachs, T. 1989. The development of vascular networks during leaf development. Curr. Top. Plant Biochem. Physiol. **8**: 168–183.

Sawchuk, M.G., Head, P., Donner, T.J., and Scarpella, E. 2007. Time-lapse imaging of *Arabidopsis* leaf development shows dynamic patterns of procambium formation. New Phytol. **176**(3): 560–571. doi:10.1111/j.1469-8137.2007.02193.x. PMID:17953541.

Sawchuk, M.G., Donner, T.J., Head, P., and Scarpella, E. 2008. Unique and overlapping expression patterns among members of photosynthesis-associated nuclear gene families in *Arabidopsis*. Plant Physiol. **1148**(4): 1908–1924. doi:10.1104/pp.108.126946. PMID:18820083.

Scarpella, E., Francis, P., and Berleth, T. 2004. Stage-specific markers define early steps of procambium development in *Arabidopsis* leaves and correlate termination of vein formation with mesophyll differentiation. Development, **131**(14): 3445–3455. doi:10.1242/dev.01182. PMID:15226260.

Scarpella, E., Marcos, D., Friml, J., and Berleth, T. 2006. Control of leaf vascular patterning by polar auxin transport. Genes Dev. **20**(8): 1015–1027. doi:10.1101/gad.1402406. PMID:16618807.

Sieburth, L.E. 1999. Auxin is required for leaf vein pattern in *Arabidopsis*. Plant Physiol. **121**(4): 1179–1190. doi:10.1104/pp.121.4.1179. PMID:10594105.

Sieburth, L.E., and Deyholos, M.K. 2006. Vascular development: the long and winding road. Curr. Opin. Plant Biol. **9**(1): 48–54. doi:10.1016/j.pbi.2005.11.008. PMID:16332447.

Steynen, Q.J., and Schultz, E.A. 2003. The *FORKED* genes are essential for distal vein meeting in *Arabidopsis*. Development, **130**(19): 4695–4708. doi:10.1242/dev.00689. PMID:12925595.

Wenzel, C.L., Schuetz, M., Yu, Q., and Mattsson, J. 2007. Dynamics of *MONOPTEROS* and *PIN-FORMED1* expression during leaf vein pattern formation in *Arabidopsis thaliana*. Plant J. **49**(3): 387–398. doi:10.1111/j.1365-313X.2006.02977.x. PMID:17217464.

Wisniewska, J., Xu, J., Seifertova, D., Brewer, P.B., Ruzicka, K., Blilou, I., Rouquie, D., Benkova, E., Scheres, B., and Friml, J. 2006. Polar PIN localization directs auxin flow in plants. Science (Wash.), **312**(5775): 883. doi:10.1126/science.1121356. PMID:16601151.

REVIEW / SYNTHÈSE

# "Genealogy" of plant physiology research in Canada and the founding of the Canadian Society of Plant Physiologists[1]

**Constance Nozzolillo**

**Abstract:** Two men were leaders in the development of plant physiology research in Canada: Professor G.W. Scarth at McGill University and Professor G.H. Duff at the University of Toronto. The latter was the driving force behind the formation of the CSPP in 1958. The contributions of these two men and their graduate students to plant physiology research in Canada are briefly summarized.

**Résumé :** On reconnaît deux chefs de file dans le développement de la physiologie végétale au Canada. Il s'agit du professeur G.W. Scarth à l'Université McGill et le professeur G.H. Duff à l'Université de Toronto. Ce dernier a joué un rôle déterminant dans la mise sur pied de la SCPV en 1958. On présente une revue des contributions de ces deux hommes ainsi que de leurs étudiants gradués à la recherche en physiologie végétale au Canada.

[Traduit par la Rédaction]

## Introduction

Students of plant physiology learn that an Englishman, the Reverend Stephen Hales (1677–1761), is the "father" of research in plant physiology. Two individuals, George William Scarth and George Henry Duff, are the "fathers" of plant physiology research in Canada.

### George William Scarth (1881–1951)

George William Scarth was born in the Orkney Islands, educated at the University of Edinburgh (he was awarded an M.A. in 1904), and hired by McGill University in Montreal in 1920 (M. Shaw, personal communication). It was there he spent the rest of his life, until his untimely death 3 months after his 70th birthday.

His research interests at McGill were many and varied. Early in his career at McGill he published papers based on ecological studies in Quebec. In the first volume of the journal *Plant Physiology* (1926), his paper entitled "Mechanisms of accumulation of dyes by living cells" appeared. This interest in cell physiology subsequently led to studies of the nature of protoplasm, stomatal mechanisms, frost resistance, tree physiology, and finally host–parasite relationships. Having grown up on a farm, he always maintained an interest in the practical applications of knowledge gained by research. His studies on wood structure and log sinkage were financially supported by the Canadian Pulp and Paper Association, and he was an influential member of the Macdonald Pasture Committee.

Many honours were bestowed on Scarth as his reputation grew in Canada, the United States, and Europe. In 1928 he was elected to the Royal Society of Canada and in 1935 he served as President of Section V. In 1933 McGill awarded him a D.Sc. In 1934, he became Macdonald Professor of Botany and Head of the Department at McGill. He was Vice-President of the American Society of Plant Physiologist (ASPP) in 1937, member of the editorial board of *Plant Physiology*, and named a Charles Reid Barnes life member of the ASPP in 1944. He retired from active teaching in 1946 but as Emeritus Professor continued work on stomatal function, and improving means of measuring photosynthesis using the infrared gas absorption method. Volume 26 of *Plant Physiology* (1951) was dedicated to him. Michael Shaw, in a lecture on Scarth given at the 1964 meeting of the CSPP, stated that a British botanist had told him that Scarth's name was a household word in Britain.

Although Scarth himself did not live to see the establishment of the CSPP, he was fully in support of the idea as evidenced by a letter written to Duff in 1950 (Bidwell and Gorham 1983).

### George Henry Duff (1893–1958)

George Henry Duff was born in China, where his parents presumably were missionaries (Forward 1968). The family returned to Hamilton, Ontario, in 1907 and George attended high school in that city. He graduated with a B.A. in 1916, an M.A. in 1917, and a Ph.D. in 1922, all from the Univer-

Received 31 October 2008. Published on the NRC Research Press Web site at botany.nrc.ca on 15 July 2009.

**C. Nozzolillo.** Department of Biology, University of Ottawa, Ottawa, ON K1N 6N5, Canada (e-mail: cnozzoli@rogers.com).

[1]This paper is one of a selection published in a Special Issue comprising papers presented at the 50th Annual Meeting of the Canadian Society of Plant Physiologists (CSPP) held at the University of Ottawa, Ontario, in June 2008.

 doi:10.1139/B09-010 

sity of Toronto. He began his long career at his alma mater in 1920 when he was hired as a lecturer in plant physiology. Plant physiology was taught from 1909 to 1919 by C.D. Howe, who held a Ph.D. in ecology from the University of Chicago, but the professor who guided Duff through his Ph.D. studies (awarded 1922, thesis *The development of the Geoglossaceae* (Mills 1968)) was probably the botanist Robert Boyd Thompson, who researched the anatomy and morphology of vascular plants.

Duff was chiefly responsible for the development of research in plant physiology in the Botany Department at the University of Toronto. His research was concerned mainly with changing metabolic patterns in growing and developing tissues, and with controls of respiratory metabolism. From 1929–1931, Duff was commissioned by the National Research Council of Canada (NRC) to investigate the damage to crops and forests resulting from the operation of a lead–copper smelter at Trail B.C., in the Canadian Rocky Mountains, and he co-ordinated and presented expert evidence on behalf of Canada before the International Joint Commission meeting to assess damages in Canada and the US. His expertise was later called upon concerning litigation associated with smoke damage in other places. Duff was appointed a full professor in 1941, after 13 years as as associate professor.

During World War II (WWII), natural rubber was in short supply. The Canadian Department of Agriculture (CDA) and the NRC undertook a country-wide programme to investigate the possibility of producing natural rubber in Canada, and the botany departments of various universities were recruited to help. Duff organized research involving several staff members, graduate and undergraduate students, and technical assistants. Their work concentrated primarily on the Russian dandelion (*Taraxacum kok-saghyz* Rodin) with some research on native milkweed (*Asclepias syriaca* L.) as rubber sources. The work resulted in rubber obtained from Russian dandelion that was used for a test batch of oxygen masks required by the Air Force. It proved superior to any synthetic rubber then available. In 1946, Duff undertook a study of the growth and reproduction of red pine (*Pinus resinosa* Ait.) in Canada at the request of the Ontario Department of Lands and Forests. The single project that had the greatest impact was the study of wood growth in pine trees, which he initiated. As stated by Dorothy Forward (Forward 1968) "This introduced a new and revealing approach to the analysis of tree growth that attracted world-wide attention." The study was later transferred to the Forest Biology division of the Canadian government as an extra-mural research project.

Duff was the virtual founder of the CSPP. In 1950 he organized a colloquium on plant physiology in Toronto that was the first of eight in succeeding years. Plant physiologists from across the country attended these annual meetings of which he was a central figure. He became chairman of an executive committee of plant physiologists that arranged these and ultimately organized a formal society. Unfortunately, he died in 1958 just a few weeks before the inaugural meeting of a fully-fledged and active CSPP with 86 founding members.

## Scarth's students

Scarth's interest in frost hardiness was pursued by three doctoral students: Jacob Levitt, who took a position at an American University after graduation and so will not be given further consideration; David Siminovitch, who is discussed below; and A.O. Whiteside, who joined the CDA in Ottawa but was never a member of the CSPP and is therefore not considered further.

### *David Siminovitch (1916–2001)*

David Siminovitch obtained his Ph.D. in 1938 (Burke and Nozzolillo 2002). Siminovitch, Levitt, and Scarth demonstrated that disruption of the plasma membrane was the primary cause of freezing injury in plants. Siminovitch and Levitt showed the importance of plasma membrane permeability in avoiding intracellular ice, and showed how plasma membrane permeability increased during cold acclimation. More importantly, they showed that extracellular ice caused cell dehydration, which also could lead to disruption of the plasma membrane and "deplasmolysis injury" during thawing. This is now recognized as one of the key mechanisms of frost injury to cold-hardy plant cells. Siminovitch, Levitt, and Scarth's early insights into the involvement of plasma membrane disruption in the mechanism for freezing damage were remarkable for their time and would wait almost 40 years to be fully appreciated.

Siminovitch went on to obtain a second Ph.D. in agricultural biochemistry at the University of Minnesota in 1948 with D.R. Briggs as advisor. There he initiated a series of studies of the biochemistry of trees with particular emphasis on the plasma membrane during winter acclimation. He used the black locust (*Robinia pseudoacacia* L.) as a model system and this work continued long after he joined the CDA in 1950 as a research scientist, ultimately heading the winter hardiness section of the Chemistry and Biology Research Institute. In addition he held the position of adjunct professor at Carleton University.

Siminovitch was a founding and life-long member of the CSPP. Among the many awards he received in recognition of his work are the CSPP Gold Medal in 1972, election as Fellow of the Royal Society of Canada in 1973, and the Board of Governor's award from the Cryobiology Society in 1987. In his Gold Medal address at the joint ASPP/CSPP meeting in Calgary in 1973, he described his work with J.W. Butler of the Laurentian Concentrates Company. They had developed a protective foam that could be laid down over tender plants when overnight freezing temperatures were expected and thereby lengthen the growing season.

### *William Harold Minshall (1911–2007)*

William Harold Minshall earned both an M.Sc. (in 1938) and a Ph.D. (awarded in 1941, thesis *Effects of acidity on growth, structure and physiology of plants with special reference to root cells*) under Scarth's tutelage while on leave from his position in the Division of Botany in the CDA at the Central Experimental Farm (CEF) in Ottawa (G. Sinclair, personal communication). He later brought the infrared gas analysis methodology that Scarth developed to the CEF in Ottawa and used it in studies of the herbicidal effects of oils. The results of these studies were presented at the first of the plant physiology colloquia in 1950 (Bidwell and Gorham 1983, Appendix I: W.H Minshall and V.A Helson). The author remembers ducking the low-hanging pipes in the basement of the now-demolished greenhouse header-

house in the arboretum in 1950–1951 to use the device when she was working there as a newly graduated M.A. and Agricultural Research Officer, Grade One. Minshall transferred to the Agriculture Research Station in London, Ontario, in 1951 and spent the rest of his long career there, retiring in 1975. He was a founding and life-long member of the CSPP. In 1983, he was recipient of the Distinguished Agrologist Award and was a Fellow of the Agriculture Institute of Canada, Canadian Pesticide Management Society, American Association for the Advancement of Science (AAAS), and Weed Society of America. In addition to his research on herbicides, he also gave lectures in plant physiology at the University of Western Ontario.

### *Herb Stern*

Herb Stern (Ph.D. awarded from McGill University in 1945) began his seminal studies of cell division under Scarth and Professor Huskins, and worked for a time at the CEF before taking up an academic position at the University of Illinois. He was a founding member of the CSPP.

### *G.H. Neil Towers (1923–2004)*

Neil Towers obtained a Master's degree as a student of Darnley Gibbs, Scarth's colleague and collaborator in research (Guy 2005). After obtaining his Ph.D. at Cornell in 1954, Towers returned to Canada to become a professor at McGill, then moved to NRC in Halifax, and finally to Vancouver and the University of British Columbia where he spent the remainder of his long and distinguished career. There he was Head of the Department of Botany from 1964–1971. He was a founding and life-long member of the CSPP, its president in 1965, and recipient of the CSPP Gold Medal in 1973. He was elected a Fellow of the Royal Society of Canada, which awarded him the Flavelle Medal in 1986. In 2000, he was awarded the Pergamon Phytochemistry Prize and in 2001 was recognized as one of the world's most highly cited scientists by ISI. During his long and productive career, which he continued unabated after his mandatory retirement at age 65, he supervised many graduate students and post-doctoral fellows (PDFs), published over 425 papers and book chapters, and travelled extensively to collect plants for his studies. Ragai Ibrahim, his first graduate student, was awarded the CSPP Gold Medal in 2004.

### *Michael Shaw*

Born in 1924, Michael Shaw was the last of Scarth's students, obtaining his Ph.D. in 1949 (thesis title: *Mutual and environmental relationships of transpiration, photosynthesis and stomatal opening*) (M. Shaw, personal communication). After a post-doctoral period in England, he joined the Department of Biology at the University of Saskatchewan in 1950 where he remained for 17 years, the final six (1961–1967) as Department Head. In 1952 he was persuaded by the University President to undertake research on the physiology of the rust fungi, although still maintaining an interest in stomatal function. His student, Gordon Maclachlan (M.Sc. awarded in 1954) worked on stomatal function, whereas all 12 of his Ph.D. students and 15 Master's students pursued ground-breaking studies of host–parasite relationships. Maclachlan obtained a Ph.D. degree at the University of Manitoba and became a professor at McGill. He is renowned for his research on cell-wall biochemistry. He was president of the CSPP in 1973 and was awarded the CSPP Gold Medal in 1985.

In 1967, Shaw moved to the University of British Columbia as Dean of Agricultural Sciences. From 1975 to 1981 he was Vice-President Academic, then Vice-President and Provost from 1981 to 1983. Until his retirement in 1989 he held the honoured title of University Professor. He was a founding member of the CSPP, as were his students T.G. Atkinson, and N. Colotelo, and remains a member today. He served as its President in 1963, and was the second recipient of the CSPP Gold Medal in 1971. He also received the first Gold Medal awarded by the Biological Council of Canada. He is a Fellow of the Royal Society of Canada and was awarded its Flavelle Medal in 1976. He was elected Fellow of the Canadian Phytopathological Society, the American Phytopathological Society, the New York Academy of Sciences, and the Linnean Society of London. McGill University (in 1975), the University of British Columbia (in 2003), and the University of Saskatchewan (in 2008) awarded him an honorary doctorate. Shaw's doctoral students include the 1988 CSPP Gold Medal awardee, the late Ann Oaks, life-long member of the CSPP, who spent most of her distinguished career as Professor of Biology at McMaster University, and whose research concentrated on nitrogen metabolism. Upon retirement from McMaster, she moved to the University of Guelph.

## Duff's students

The following details some of the students taught by George Henry Duff (only some of the following were Duff's doctoral students, but all were influenced by his teaching and example).

### *Dorothy F. Forward (1903–1993)*

Dorothy F. Forward was born in Iroquois, Ontario, and obtained her B.A. in biology at the University of Toronto in 1926, presumably studying plant physiology in Duff's class (Anon. 1996). However, her Ph.D. was in plant pathology (thesis awarded in 1931, *The effects of prolonged darkness upon infection type and development in stem rust of wheat*, (Mills 1968)). Like David Siminovitch, she left Canada to obtain a second Ph.D., this time in plant physiology at the University of Cambridge. She returned to the University of Toronto as a professor in 1934 where she remained until her retirement in 1971, training many graduate and undergraduate students in botanical subjects, especially plant physiology. She, Professor Duff, and students J. Scorgie and V.C.J. Quesnel, presented two papers on the results of a study of respiration in isolated maize root tips at the 1950 colloquium (Bidwell and Gorham 1983, Appendix 1). Forward continued Duff's study of red pine following his death in 1958. Forward was a founding and life-long member of the CSPP, its president in 1962 and, in 1990, the first recipient of the CSPP Gleb Krotkov Award.

### *Gleb Krotkov (1901–1968)*

Gleb Krotkov was a refugee from the Russian Revolution with a B.Sc.Agr. from Charles University in Czechoslovakia, and was working as a farm labourer in Vineland, Ontario, when he encountered Professor Duff who persuaded

him to become a student at the University of Toronto (Gorham 1968). Krotkov graduated with a Master's degree in 1931 and joined the staff at Queen's University in Kingston, continuing his studies with Duff towards a Ph.D. (awarded in 1934, thesis *Carbohydrate and respiratory metabolism in the isolated starving leaf of wheat* (Mills 1968)). During World War II (WWII), Krotkov worked on the Russian dandelion rubber project, his Russian mother tongue making him invaluable as interpreter of the Russian scientific literature on the subject. Following the war, he spent a sabbatical year in California with D.M. Hoagland and subsequently established the first radioisotope laboratory for biological research at a Canadian university. During his long career at Queen's, many students came under his supervision, including the author (and founding member of the CSPP) who received her M.A. in 1950. Those who had received a B.A. and M.A. at Queen's University, Kinston, earned a Ph.D. at Queen's only after spending a year at another university. (The author joined the CDA in Ottawa instead, and received a Ph.D. 13 years later from the University of Ottawa). In cooperation with his students, PDFs, and visiting scientists, Queen's University, Kingston, became a centre of research in photosynthesis and translocation.

Krotkov was a founding member of the CSPP, together with his students R.G.S. (Tony) Bidwell (awarded a Ph.D. in 1954), J.S. Craigie (awarded a Ph.D. in 1959), V.A. Helson (awarded an M.A. in 1942), C.D. Nelson (awarded a Ph.D. in 1955), C. Nozzolillo, (awarded an M.A. in 1950), and D.G. Wilson (awarded an M.A. in 1950). Wilson and Krotkov presented a paper at the first of Duff's colloquia in 1950 (Bidwell and Gorham 1983, Appendix I). Two of Krotkov's students achieved the position of President of the CSPP: Bidwell in 1972, and Craigie in 1982. Krotkov was president of the CSPP in 1960 and would certainly have been a recipient of the CSPP Gold Medal had he lived long enough. Instead, Bidwell (his student), was awarded the medal in 1979. In his honour, the CSPP named its award for service to the Society, established in 1988, the Gleb Krotkov Award. Krotkov spent his second sabbatical year in 1955–1956 at Harvard on a Guggenheim Fellowship with CSPP founding member K.V. Thimann, a year that introduced him to the phenomenon of photorespiration. He was elected a Fellow of the Royal Society of Canada in 1950, and awarded the Flavelle Medal in 1964. In 1962, he was appointed a Member of the NRC, a term renewed for a second 3 years in 1965, and served on at least six of the Council's standing committees and as one of the NRC representatives on the Canadian Committee for the International Biological Programme.

### *Kenneth A. Clendenning (1915–1972)*

Kenneth A. Clendenning graduated from Duff's laboratory with a Ph.D. in 1941 (thesis *Physiological studies of the tomato fruit in relation to its development and storage* (Mills 1968)). He was hired by the NRC in Ottawa where he continued studies on stored fruit until the end of WWII (Gorham and Nozzolillo 2006). After the war, his research interests turned to fundamental studies of the process of photosynthesis, the results of which he presented at the first colloquium that Professor Duff organized in 1950 (Bidwell and Gorham 2983, Appendix 1). Duff then persuaded him to organize the second such colloquium in Ottawa in 1951. However, he left the NRC and moved to the US before the CSPP was formed

### *D.W. Alan Roberts (1921–2001)*

Alan Roberts was born in England, but from the age of four, grew up in Toronto and graduated from the University of Toronto (M. Roberts, personal communication). During WWII he served with the Royal Canadian Navy but returned to university for post-graduate study as soon as the war ended, graduating with a Ph.D. (awarded in 1948, thesis *The metabolic basis of physiological heterogeneity in the cells of a linear organ: polarity in the first leaf of the wheat plant* (Mills 1968)). Following graduation, he accepted a research position with the CDA and moved to the Lethbridge Research Station. During his career there, he published 48 research papers plus numerous reports. He was a founding member of the CSPP and participated in the first of Duff's colloquia with a talk entitled "Distribution of invertase activity in the wheat leaf" (Bidwell and Gorham 2983, Appendix 1).

### *E. Roy Waygood (1918–2001)*

Roy Waygood was a founding and life-long member of the CSPP, its first vice-president, and its second president (Shaw 1987; Gorham 2001). He was born in England but graduated from the Ontario Agricultural College in 1941. He then completed his Master's degree (1947) and Ph.D. (awarded in 1949, thesis *Respiratory enzymes in wheat. I. The malic dehydrogenase of wheat seedlings. II The oxidases. III The respiratory carrier function of ascorbic acid* (Mills 1968)) at the University of Toronto after service with the Royal Canadian Air Force (RCAF) during WWII. He delivered the results of his graduate research in two presentations at the first of Professor Duff's colloquia in 1950 (Bidwell aand Gorham 1983, Appendix I). He spent five years at McGill University, where CSPP founding member Roger Paquin was his Ph.D. student and CSPP founding member Dalton Wang was a PDF, but Waygood was associated with the University of Manitoba for most of his career, retiring in 1979. He was a Fellow of the Chemical Institute of Canada and was elected a Fellow of the Royal Society of Canada in 1971. The CSPP president of 1973, Gordon Maclachlan, was his Ph.D. student and CSPP founding member K. Lakshminarayan was his PDF at the University of Manitoba. John King, CSPP president in 1983 and CSPP Gold Medal awardee in 2001, also obtained his Ph.D. under Waygood's tutelage.

### *Donald A. Fraser (1918–2003)*

Donald A. Fraser, a native of Toronto and graduate of the University of Toronto, was a bomber pilot during WWII and returned to the University of Toronto after the war, obtaining a Ph.D. (awarded in 1951, thesis *A study of cambial activity* (Mills 1968)) (E. Gaertner, personal communication). His thesis supervisor was Professor H.B. Sifton, but Duff evidently had a major influence on his research activities. Following graduation, Fraser worked for a time with the CDA in Sault Ste. Marie, before moving to Petawawa where he worked for the Canadian Forestry Service and pioneered the use of radioactive isotopes in forest tree research. Upon closure of the Petawawa Station, he moved to Concordia

University as Chair of Geography, continuing forestry research with ecological studies of spruce trees in the James Bay region. He was a founding member of the CSPP.

***Gordon M. Ward (1912–2005)***

Gordon M. Ward was born in Brampton, Ontario, and obtained a B.Sc. at the University of Saskatchewan (1932), and an M.A. from McMaster University (1933), after which he joined the CDA in Ottawa as a tobacco physiologist (J. Traquair and D. Drew, personal communication). He served in the RCAF from 1941–1945. He received a Ph.D. from the University of Toronto in 1952 (thesis *The metabolism of sucrose in the wheat leaf* (Mills 1968)) and returned to the CEF in Ottawa following graduation. In 1959, he transferred to the CDA lab at Harrow, Ontario, where his work and that of his colleagues was the foundation for the modern multi-million dollar greenhouse industry in southern Ontario. He was a founding member of the CSPP, elected a Fellow of the CIC in 1976, and published 80 research papers.

***Frank Forsyth (1922–1996)***

Frank Forsyth graduated from Queen's with an B.A. (Hons.) in 1949 after service with the RCAF as a Spitfire pilot during WWII, and continued his studies to a Ph.D. at the University of Toronto (awarded in 1952, thesis *Germinative growth and respiration in the seed of* Pinus resinosa *(Ait) in relation to oxygen supply* (Mills 1968)) (J. Forsyth-Lee, personal communication). He spent his working life with the CDA, first in Winnipeg for 6 years, then in London, Ontario, for 3 years, and finally in Nova Scotia where he worked on apple physiology. He was a founding and long-time member of the CSPP, and filled the role of President of the CSPP in 1967.

***R.G.S. (Tony) Bidwell***

Born in 1927, Tony Bidwell was a student in Duff's laboratory in 1953 working to fulfill the one-year-away-requirement at Queen's for a Ph.D. degree, and returned to Toronto as Associate Professor of Botany in 1959 after 3 years with the NRC in Halifax, Nova Scotia. In 1965, he moved to Case-Western Reserve University in Ohio as Professor and Chair of Biology, returning to Queen's in 1969 as Professor of Biology (R.G.S. Bidwell, personal communication). He took early retirement in 1979, but remained very active in scientific research as, among other activities, Killam Research Professor at Dalhousie University (1981–1985), Lansdowne Visiting Professor of Biology at Mt. Allison University (1988–1991), and Visiting Professor at the University of Victoria (1992). The first edition of his textbook *Plant Physiology* was published in 1974 and the second edition in 1979. The list of scientific publications as sole author, or with graduate students and colleagues, includes over 150 titles. Honours include, in addition to the CSPP Gold Medal (1979) and the Queen Elizabeth II Silver Jubilee Medal (1977), induction into the Royal Society of Canada in 1979 and as a Fellow of the AAAS in 1987.

***A.R.A. Taylor***

A.R.A. Taylor (Ph.D. from the University of Toronto, awarded in 1955, thesis *A developmental study of* Zostera marina *L.* (Mills 1968)) was a founding member of the CSPP, but his interests in algae later made the Canadian Botanical Association his society of choice. Following graduation from the University of Toronto, he joined the University of New Brunswick where he remained until retirement.

## CSPP

The CSPP came into existence in 1958 and has thrived ever since, celebrating its 50th anniversary in 2008 at the annual meeting in Ottawa. Its membership includes graduates from the laboratories of Scarth and Duff's students who populated university campuses and government research laboratories across the country. For example, the late C.D. Nelson left his associate professor post at Queen's in 1965 to become Chairman of the Biology Department at the newly founded Simon Fraser University, and later, Dean of the Science Faculty. Like his mentor Krotkov, Nelson would certainly have been a recipient of the CSPP Gold Medal had he not succumbed to leukemia in 1968. In his memory, the CSPP established the C.D. Nelson Award in 1977 to recognize excellence in research by young scientists. Bruce Tregunna would very likely have been the first recipient of this award had he not suffered a fatal heart attack in 1975. His Ph.D. thesis (awarded in 1963) contains the first modern definition of photorespiration as being a high oxygen-dependent carbon dioxide emission during photosynthesis (P. Joliffe, personal communication). Tregunna went to McGill University in 1964 and then followed Towers to the University of British Columbia in 1965. In recent years, membership has remained at a steady level of about 400. The ranks of membership, of course, are filled not only by students of Duff and Scarth but also by students of others, both those who were born in Canada (e.g., the late Paul Gorham) or immigrants such as those who arrived in Canada as PDFs funded by the NRC, and who remained to take positions at Canadian universities. However, for brevity, only some of those with links to Scarth or Duff are considered in the present historical perspective. Francophone members have made many invaluable contributions to the development of the CSPP; six were founding members and many have served as members of the Executive Committee and other committees. Their importance to the CSPP is recognized by efforts to provide bilingual publications whenever possible and by the dual name: Canadian Society of Plant Physiologists / La Société Canadienne de Physiologie Végétale, CSPP/SCPV. Only one francophone is named, however, Roger Paquin, CSPP president in 1977, because he was both a founding member and had been a student of Waygood.

## Acknowledgements

The author is grateful to the following for information otherwise not readily available: Michael Shaw for mailing his original manuscript for the talk on Scarth he gave at the 1964 CSPP national meeting together with additional material on Scarth's and his own career; Tony Bidwell for e-mailing his curriculum vitae; Robert Van Huystee for putting me in contact with Harold Minshall's daughter, Gaye Sinclair; Erika Gaertner for details of the career of her late husband, Don Fraser, and similarly the widows of Alan Roberts and Frank Forsyth; Peter Joliffe for information on his Ph.D. supervisor,

Bruce Tregunna; and finally, Agriculture and Agri-Food Canada employees Jim Traquair, Dorothy Drew, and Andre Laroche for input on the careers of Gordon Ward and Alan Roberts.

## References

Anonymous 1996. *In* Memorium: Forward was a phenomenol teacher. The Bulletin (24 June) University of Toronto Press, Toronto, Ont.

Bidwell, R.G.S. and Gorham, P.G. 1983. The first 25 years, A history of the Canadian Society of Plant Physiologists / La Société Canadienne de Physiologie Végétale. [Online]. Available from www.cspp-scpv.ca/archives-e.shtml.

Burke, M.J., and Nozzolillo, C. 2002. *In* Memorium: David Siminovitch (1916–2001). Cryobiology, **44**: 1–3. doi:10.1016/S0011-2240(02)00006-8.

Forward, D. 1968. The History of the Botany Department at the University of Toronto. University of Toronto Press, Toronto, Ont.

Gorham, P.R. 1968. Gleb Paul Krotkov 1901–1968. *In* Proceedings of the Royal Society of Canada. Available from www.rsc.ca.

Gorham, P.R. 2001. E. Roy Waygood (1918–2001). Bulletin of the Canadian Society of Plant Physiologists / Société Canadienne de Physiologie Végétale. Available through CSPP archivist, C. Nozzolillo.

Gorham, P.R., and Nozzolillo, C. 2006. Photosynthesis research in Canada from 1945 to the early 1970s. Photosynth. Res. **88**: 83–100. doi:10.1007/s11120-005-9022-z. PMID:16447031.

Guy, R. 2005. George Hugh Neil Towers (1923–2004). Bulletin of the Canadian Society of Plant Physiologists / Société Canadienne de Physiologie Végétale (April issue). Available from www.cspp-scpv.ca.

Mills, J. 1968. University of Toronto doctoral theses, 1897–1967: a bibliography. University of Toronto Press, Toronto, Ont.

Shaw, M. 1987. A tribute to Professor E.R. Waygood. Bulletin of the Canadian Society of Plant Physiologists / Société Canadienne de Physiologie Végétale. Available through CSPP archivist, C. Nozzolillo.

# Overexpression of a directed mutant of 14-3-3ω in *Arabidopsis* leaves affects phosphorylation and protein content of nitrate reductase[1]

**Man-Ho Oh, Joan L. Huber, Wei Shen, Gurdeep S. Athwal, Xia Wu, and Steven C. Huber**

**Abstract:** The 14-3-3 family of proteins are highly conserved signaling proteins in eukaryotes that bind to their client proteins, usually through specific phosphorylated target sequences. While the 14-3-3 proteins are thought to interact with a wide array of cellular proteins, there have been few studies addressing the in-vivo role of 14-3-3. As one approach to study this in-vivo role, we generated transgenic *Arabidopsis* plants constitutively overexpressing a directed mutant of 14-3-3 isoform ω that inhibits phosphorylated nitrate reductase (pNR) in a largely divalent-cation-independent manner in vitro. The transgenic plants had increased relative phosphorylation of NR at the regulatory Ser-534 site and decreased NR activity measured in the presence of 5 mmol·$L^{-1}$ $MgCl_2$ relative to nontransgenic plants. In addition, total NR protein was increased and the protein half-life was increased about two-fold. Two-dimensional difference gel electrophoresis analysis of proteins extracted from leaves of plants expressing the mutant 14-3-3 identified numerous cellular proteins that were altered in abundance. In particular, several β-glucosidase and glutathione *S*-transferase isoforms were decreased in abundance relative to wild type plants suggesting a possible alteration in stress or defense responses.

*Key words: Arabidopsis thaliana*, 14-3-3 protein, β-glucosidase, glutathione *S*-transferase, two-dimensional difference gel electrophoresis, site-directed mutagenesis.

**Résumé :** Les protéines 14-3-3 constituent des protéines de signalisation fortement conservées, chez les eucaryotes, qui se lient généralement à leurs protéines clientes par des séquences phosphorylées spécifiquement ciblées. Bien qu'on croit que les protéines 14-3-3 interagissent avec un large ensemble de protéines cellulaires, il existe quelques études sur le rôle in vitro des 14-3-3s. Comme approche à cette étude, les auteurs ont généré des plants d'*Arabidopis* transgénique surexprimant constitutivement un mutant dirigé de l'isoforme ω des 14-3-3, lequel inhibe la nitrate réductase phosphorylée (pNR) de façon largement indépendante de cations divalents in vitro. Les plantes transgéniques montrent une augmentation relative de la phosphorylation de la NR au site de régulation Ser-534 et diminuent l'activité de la NR mesurée en présence de $MgCl^2$ 5 mmol·$L^{-1}$, par rapport aux plantes non transformées. De plus, on observe une augmentation de la protéine NR totale et la demi-vie des protéines est à peu près doublée. L'analyse électrophorétique des différences sur gel à deux dimensions des protéines extraites des feuilles des plantes exprimant le mutant 14-3-3, fait ressortir de nombreuses protéines cellulaires dont l'abondance est fortement altérée. En particulier, plusieurs isoformes des β-glucosidases et gluthation-*S*-transférases diminuent en abondance par rapport aux plantes de type sauvage, ce qui suggère une possible altération des réactions de défenses au stress.

*Mots-clés : Arabidopsis thaliana*, protéine 14-3-3, β-glucosidase, gluthation-*S*-transférases, analyse électrophorétique des différences sur gel à deux dimensions, mutagenèse ciblée.

[Traduit par la Rédaction]

Received 2 November 2008. Published on the NRC Research Press Web site at botany.nrc.ca on 10 July 2009.

**M. Oh and S.C. Huber.**[5] US Department of Agriculture – Agricultural Research Service, and Departments of Plant Biology and Crop Sciences, University of Illinois, Urbana, IL 61801, USA.
**J.L. Huber.**[2] Department of Plant Biology, University of Illinois, Urbana, IL 61801, USA.
**W. Shen**[3] **and G.S. Athwal.**[4] US Department of Agriculture-Agricultural Research Service, NC State University, Raleigh, NC 27695-7631, USA.
**X. Wu.** Program in Physiological and Molecular Plant Biology, University of Illinois, Urbana, IL 61801, USA.

[1]This paper is one of a selection published in a Special Issue comprising papers presented at the 50th Annual Meeting of the Canadian Society of Plant Physiologists (CSPP) held at the University of Ottawa, Ontario, in June 2008.
[2]Present address: Department of Molecular and Cellular Biology, University of Illinois, Urbana, IL 61801, USA.
[3]Present address: Department of Structural and Molecular Biology, North Carolina State University, Raleigh, NC 27695, USA.
[4]Present address: Novozymes Biopharma UK Ltd, 59 Castle Boulevard, NG7 1FD Nottingham, UK.
[5]Corresponding author (e-mail: schuber1@illinois.edu).

## Introduction

The 14-3-3 proteins are ubiquitous signaling proteins in eukaryotes that bind to many cellular client proteins, usually in a phosphorylation-dependent manner (Muslin et al. 1996; MacKintosh 2004). Thus, 14-3-3:client interactions are controlled to a large extent by phosphorylation of the client protein, as mediated by signals controlling the requisite kinase(s) and phosphatatase(s). In addition, at least some 14-3-3:client interactions may also be affected by divalent cations (Shen and Huber 2006). Initial studies with 14-3-3ω established that the protein could bind $Ca^{2+}$ and $Mg^{2+}$ in the C-terminal portion of the protein, and identified loop 8 as a potential EF-like hand sequence that could be involved in cation binding (Lu et al. 1994). About the same time, the inhibition of phosphorylated nitrate reductase (pNR) by its "inhibitor protein" was demonstrated and shown to be dependent on millimolar concentrations of divalent cations (Glaab and Kaiser 1995, 1996). Interestingly, phosphorylation of NR at the Ser-534 regulatory site (Douglas et al. 1995; Bachmann et al. 1996*a*; Bachmann et al. 1996*c*; Su et al. 1996) has no direct effect on catalytic activity, but rather is required to complete the binding site for an inhibitor protein that was subsequently identified as a 14-3-3 protein (Bachmann et al. 1996*b*; Moorhead et al. 1996). The interaction between pNR and the 14-3-3 protein was shown to be strongly stimulated by divalent cations in vitro (Athwal and Huber 2002), providing an explanation for the earlier results of Glaab and Kaiser (1995). Similarly, binding of 14-3-3ω to a synthetic peptide based on the regulatory phosphorylation site of NR was also stimulated by divalent cations (Athwal et al. 2000). An extensive analysis of the interactions of 10 recombinant *Arabidopsis* 14-3-3 isoforms with phosphopeptides based on the binding sites derived from NR (a mode 1 site) or the plasma membrane proton pump (AHA2; a mode 3 site) demonstrated that the binding activity of each of the isoforms to both targets was enhanced by calcium or magnesium, although quantitative differences were noted (Manak and Ferl 2007). Hence, the overall conclusion to emerge is that 14-3-3 binding to target polypeptides may be broadly regulated by divalent cations. Consistent with this notion is the fact that the loop 8 sequence of the 14-3-3 protein that has been identified as the likely cation-binding site (Lu et al. 1994; Athwal and Huber 2002) is highly conserved across isoforms. In *Arabidopsis* 14-3-3ω, an acidic residue at the beginning of loop 8, Glu-208, was found to play a key role in regulation of 14-3-3 function by divalent cations. Substitution of Glu-208 with an alanine (the 14-3-3$\omega^{E208A}$ directed mutant) resulted in a 14-3-3 protein that produced significant inhibition of pNR activity in the absence of divalent cations and also stronger inhibition of the activity of pNR compared with wild type 14-3-3ω, indicating an increase in overall binding affinity (Athwal and Huber 2002). The altered properties of the E208A mutant suggested possible utility for examining the role of cations in regulating 14-3-3:client interactions in vivo and this was the starting point for the present study.

It is worth noting that loop 8 also plays a role in isoform specificity in client interactions that appears to be independent of cation regulation. In barley, for example, 14-3-3A and 14-3-3B are the only two characterized isoforms that are expressed in leaves and only 14-3-3B is able to inactivate pNR (Sinnige et al. 2005). Interestingly, a major factor determining the ability to interact with pNR is a single residue in loop 8; 14-3-3A has a serine whereas 14-3-3B has a glycine residue. Substituting the serine with glycine in 14-3-3A dramatically increased the ability to inhibit pNR activity and also allowed the directed mutant to bind to a synthetic phosphopeptide based on the NR regulatory phosphorylation site (Sinnige et al. 2005). Another example involves mammalian 14-3-3σ wherein three residues in loop 8 that are unique to this isoform restrict ability to bind to Cdc25C, a client of many 14-3-3 isoforms but not σ (Wilker et al. 2005). Thus, loop 8 plays a critical role in several aspects of 14-3-3 functionality.

While much is known about 14-3-3 proteins, there is also much that remains to be investigated. For example, while many potential client proteins have been identified (Moorhead et al. 1999; Cotelle et al. 2000; MacKintosh 2004; Alexander and Morris 2006; Schoonheim et al. 2007), establishing which interactions occur in vivo and are important for plant function remains largely unexplored. Critical aspects to be identified are which proteins interact with 14-3-3s in vivo, and what the effects of binding are to the clients. The impact of 14-3-3 binding can be to alter activity (if the client is an enzyme) or subcellular localization, reduce access to protein phosphatases, or to modulate proteolytic degradation (Tzivion and Avruch 2002). In the case of NR, it is clear that 14-3-3 binding inhibits enzymatic activity and reduces dephosphorylation, but whether the pNR:14-3-3 complex is more or less susceptible to proteolytic degradation remains controversial. As one begins to address some of these questions in planta, we have generated transgenic *Arabidopsis* plants overexpressing directed mutants of 14-3-3ω with altered functionality. Herein, the impact on NR phosphorylation and NR protein content was determined by immunoblotting with specific antibodies, and experiments were conducted to investigate the impact on the cellular proteome using differential in-gel electrophoresis (DIGE) technology.

## Material and methods

### Plant material and growth conditions

*Arabidopsis thaliana* (L.) Heynh ecotype Columbia (Col-0) was used as the wild type. Plants for protein extraction and analysis were grown in a soil mixture with short days (8 h) in growth chambers (Conviron Model PGW36, Winnipeg, Manitoba, Canada) with photosynthetic photon flux density of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at plant level and day–night temperature of 22–18 °C. Fully expanded rosette leaves were harvested into liquid nitrogen from 3- to 4-week-old vegetative plants, as specified in the text. Plants were fertilized biweekly with Hoagland's solution (Hoagland and Arnon 1950).

### Electrophoresis and immunoblotting

Liquid nitrogen powdered tissue was mixed with four volumes of pre-heated (95 °C) 1× SDS–PAGE sample buffer containing 1 $mol \cdot L^{-1}$ urea, 0.7 $mol \cdot L^{-1}$ 2-mercaptoethanol, 5 $mmol \cdot L^{-1}$ NaF, 1 $mmol \cdot L^{-1}$ $Na_2MoO_4$, 1 $mmol \cdot L^{-1}$ $Na_3VO_4$, 1 $mmol \cdot L^{-1}$ aminoethylbenzenesulfonyl fluoride (AEBSF), and 2 $mmol \cdot L^{-1}$ EDTA. Protein concentrations

**Fig. 1.** Inhibition of pNR activity by 14-3-3ω and directed mutants of loop 8. (A) Schematic representation of the directed mutants tested in vitro. Activity of pNR was measured with increasing amounts of the 14-3-3 proteins in the (B) absence and (C) presence of 5 mmol·L$^{-1}$ $Mg^{2+}$ at pH 7.5, and in the absence of $Mg^{2+}$ at pH 6.5 as indicated.

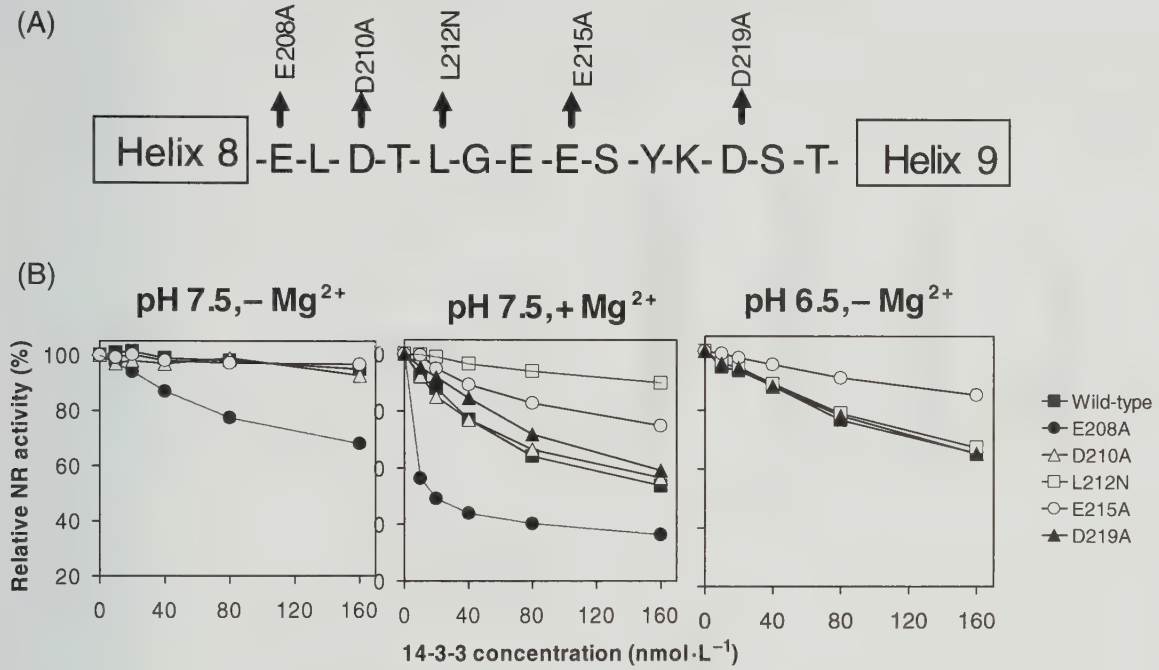
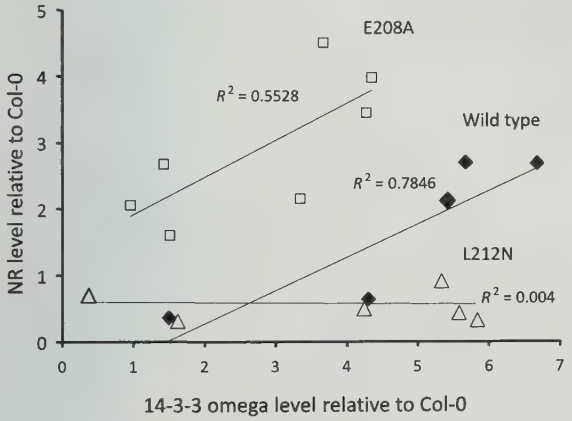
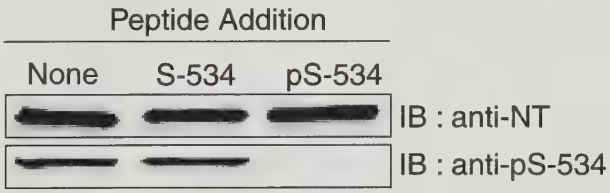



**Fig. 2.** Correlation between the over-expression of 14-3-3ω proteins and the steady-state level of nitrate reductase (NR) protein in transgenic *Arabidopsis* plants. *Arabidopsis* leaf soluble protein extracts from wild type Col-0 plants and transgenic plants expressing the wild type 14-3-3ω (diamonds) or E208A (squares) or L212N (triangles) directed mutants driven by the 35S promoter were subjected to immunoblot analysis with antibodies to the 14-3-3ω C-terminal peptide or NR N-terminal peptide. The ECL system was used for detection and densities of the bands (~110 kDa NR protein and ~30 kDa 14-3-3ω) were quantified with a densitometer and are plotted relative to the band intensities for nontransgenic plants.




were determined by the dye-binding assay (Bio-Rad, Hercules, California, USA) with bovine serum albumin as the standard. Proteins were separated on 12% polyacrylamide (0.1% SDS) gels and transferred to polyvinylidene fluoride

**Fig. 3.** Peptide competition demonstrates specificity of the phosphospecific antibodies directed against the Ser-534 site of *Arabidopsis* nitrate reductase (NR). Protein extracted from a dark leaf were resolved by SDS–PAGE, immunoblotted onto a PVDF membrane, and probed with anti-NT or anti-pS-534 antibodies. As indicated, the antibodies contained 50 μg of the pS-534 antigen peptide or the corresponding unphosphorylated peptide (S-534). Note that the recognition of pNR by the anti-pS-534 antibodies was affected only by the antigen peptide and neither peptide affected recognition of NR by the anti-NT antibodies.




fluorescence-specific membranes (Millipore, Bedford, Massachusets, USA). Polyclonal antibodies that recognize the N-terminal sequence of *Arabidopsis* NR and the regulatory phosphorylation site at Ser-S534 were produced by immunizing rabbits with two peptides (superscript number indicate residues in *At*Nia2): anti-NT, $^{11}$RLEPGLNGVVRSYK$^{24}$ and anti-pS534, $^{528}$SLKKSVpSTPFMNT$^{540}$. The antibodies were affinity purified, and in the case of the anti-pS534 antibodies this involved sequential processing over immunosorbent columns coupled with the unphosphorylated S534 peptide, followed by the phosphorylated peptide (Bethyl Laboratories, Montgomery, Texas, USA). The pS534:S534 reactivity ratio of the anti-pS534 antibodies against immobilized peptides was 99:1 by ELISA analysis (testing performed by

**Fig. 4.** Effect of 14-3-3$\omega^{E208A}$ overexpression on the phosphorylation of nitrate reductase (NR) at the Ser-534 site in the light and dark. Light samples were taken mid-morning, and after transfer of plants to the dark for 1 h. Three independent transgenic lines are compared with nontransgenic *Arabidopsis* Col-0 plants. In these experiments, total NR protein was ~1.6-fold higher in the E208A plants compared with Col-0.

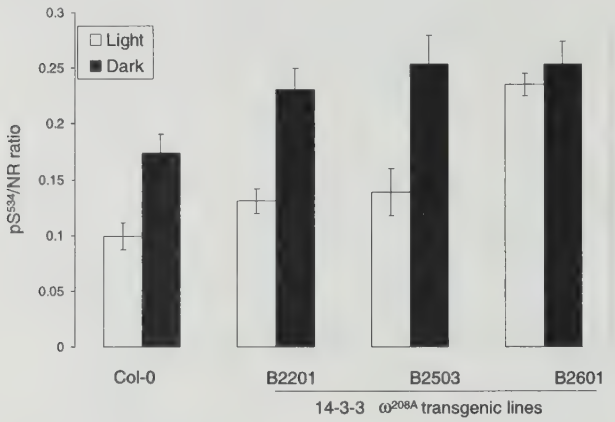
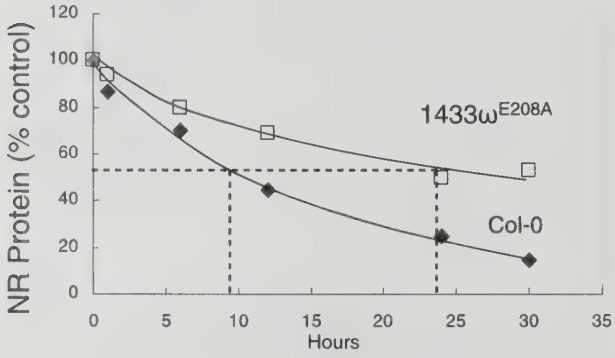



**Fig. 5.** Stability of nitrate reductase (NR) protein in leaves of nontransgenic *Arabidopsis* compared with plants overexpressing 14-3-3$\omega^{E208A}$. Detached leaves were fed 50 μmol·L$^{-1}$ cycloheximide (in water) through the cut petiole and kept in darkness for times as indicated. The initial content of NR protein and ~2-fold higher in the transgenics compared with Col-0 leaves.




Bethyl). Membranes were blocked in a 2% (*v*/*v*) fish gelatin solution in phosphate-buffered saline (PBS; 5 mmol·L$^{-1}$ $NaH_2PO_4$, 150 mmol·L$^{-1}$ NaCl, pH7.4) before being incubated with primary antibodies at 1:15 000 (anti-NT) and 1:25 000 (anti-pS534) dilutions in PBS containing 0.1% (*v*/*v*) Tween-20 (PBST). Immunoblots to detect 14-3-3ω (and the loop 8 directed mutants) used anti-CT antibodies raised against a synthetic peptide ($^{244}$DEIKEAAAPKPT$^{254}$) sequence in the COOH-terminal polypeptide of 14-3-3ω (Shen et al. 2003). Washes were performed in PBST, and an Alexa Fluor 680- or IR 800-conjugated secondary antibody was used (Rockland Immunochemicals, Gilbertsville, Pennsylvania, USA) diluted at 1:20 000 in PBST. Immunoblot imaging and densitometry analysis was performed using a LI-COR Odyssey scanner (LI-COR, Lincoln, Nebraska, USA).

### Production of 14-3-3$\omega^{E208A}$ recombinant protein

14-3-3ω constructs utilized the pET15b plasmid (Novagen, Madison, Wisconsin, USA). Production and purification of recombinant protein of GF14ω was performed as described (Athwal and Huber 2002). *Arabidopsis* cDNA in the pET15b vector was used to construct sequencing-verified, site-directed mutants (Ala substitutions) with the QuikChange XL Kit as recommended by Stratagene (La Jolla, California, USA). The E208A and D219A mutants were produced as described (Athwal and Huber 2002) and a similar protocol was used to produce the D210A, L212N, and E215A mutants. All constructs were transformed into *E. coli* BL21(DE3) for protein expression and the $His_6$-tagged proteins were affinity purified using Ni-NTA agarose (Qiagen, Hilden, Germany) following manufacturer's instructions. Protein was eluted with 50 mmol·L$^{-1}$ 4-morpholinepropanesulfonic acid (MOPS) pH 7.5, 300 mmol·L$^{-1}$ NaCl, 60 mmol·L$^{-1}$ imidazole and washed with 10 bed volumes of column equilibration buffer and dialyzed against a 1000× volume of 20 mmol·L$^{-1}$ MOPS, pH 7.5, and 1 mmol·L$^{-1}$ dithiothreitol (DTT) before use.

### Transformation of *Arabidopsis* with 14-3-3ω and directed mutants

The floral dip method for *Agrobacterium*-mediated transformation of *Arabidopsis* was performed (Clough and Bent 1998). Seeds produced were harvested and incubated with 95% EtOH for 10 min and sterilized with 30% bleach plus 0.1% Tween-20 for 20 min. After extensive washing, sterilized seeds were cold-treated for 2 d and plated onto selection media (half strength MS media containing 1% (*w*/*v*) sucrose, and 30 μg·mL$^{-1}$ kanamycin, pH 5.7) and then grown at 24 °C under long day (16 h) conditions.

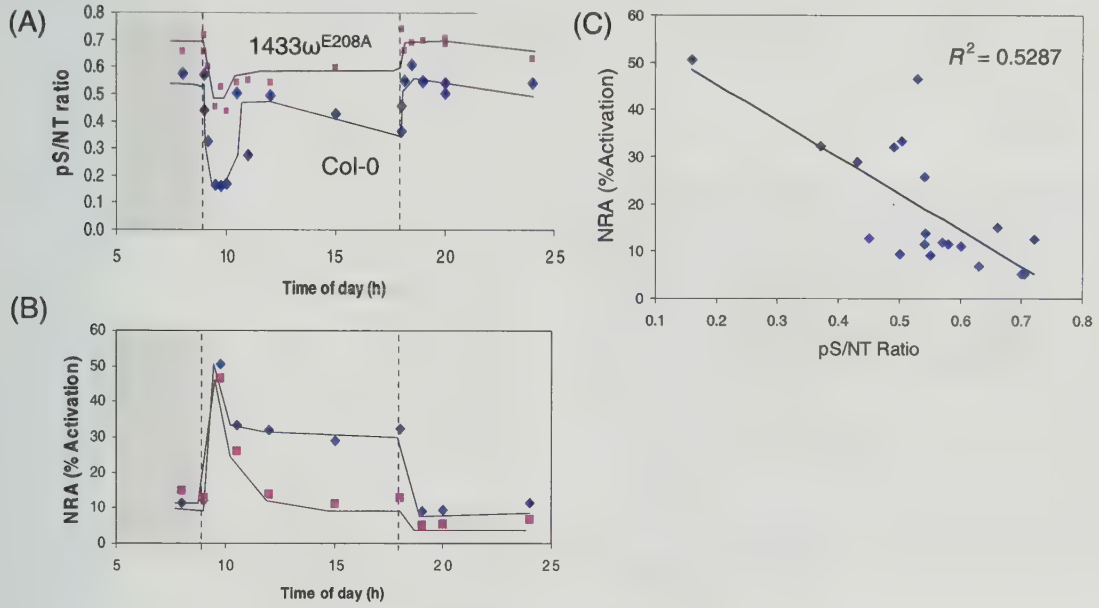
### Extraction and assay of nitrate reductase (NR) activity

Leaves were extracted and NR activity was assayed in the presence and absence of 5 mmol·L$^{-1}$ $MgCl_2$ as previously described (Shen et al. 2003).

### Extraction of total proteins for two-dimensional differential in-gel electrophoresis (DIGE)

Leaf tissue (0.5 g) was mixed with two volumes of extraction buffer (100 mmol·L$^{-1}$ Tris-HCl, pH 8.0; 2% 2-mercaptoethanol, 5 mmol·L$^{-1}$ EGTA, 10 mmol·L$^{-1}$ EDTA, 2% SDS, 1 mmol·L$^{-1}$ AEBSF, 10 μmol·L$^{-1}$ leupeptin, 1 mmol·L$^{-1}$ *para*-amino-benzamidine, 5 mmol·L$^{-1}$ caproic acid, 2 μmol·L$^{-1}$ trans-epoxysuccinyl-L-leucyl-amido (4-guanidino) butane (E64), 10 μmol·L$^{-1}$ Z-Leu-Leu-Leu-aldehyde (MG132), 0.5 μmol·L$^{-1}$ micrycystin-LR (MC-LR) and 10 mmol·L$^{-1}$ NaF) and ground in a cold mortar, and then centrifuged at 5000 r·m$^{-1}$ (2700*g*) at 4 °C for 10 min. The soluble proteins released were mixed with an equal volume of ice-cold phenol (Tris-buffered, pH 7.9) and centrifuged at 16 000 r·m$^{-1}$ (23 700*g*) for 15 min at 10 °C to separate the phenol and aqueous phases. The upper aqueous phase was discarded and the phenol phase was extracted twice more before mixing with five volumes of ice-cold 0.1 mol·L$^{-1}$ of ammonium acetate in methanol to precipitate total soluble proteins overnight at –20 °C. The protein pellet was washed three times with 1 mL aliquots of ice-cold 0.1 mol·L$^{-1}$ ammonium acetate in methanol and once with

**Fig. 6.** Diurnal changes in nitrate reductase (NR) activity in leaves of nontransgenic *Arabidopsis* Col-0 compared with plants overexpressing 14-3-3ω$^{E208A}$. (A) Relative NR phosphorylation at the Ser-534 site normalized to the amount of NR protein. Total NR protein was highest at the beginning of the photoperiod and was ~1.6-fold higher in the transgenic compared with nontransgenic plants. (B) NR activation (NRA) state, expressed as the ratio of activities measured in the presence versus absence of 5 mmol·L$^{-1}$ $MgCl_2$. (C) Negative correlation between NRA and relative phosphorylation at the Ser-534 site. Values from (B) are plotted.




ethanol before resuspension in 300 μL DIGE buffer (30 mmol·L$^{-1}$ Tris-HCl, pH 8.5, 7 mol·L$^{-1}$ urea, 2 mol·L$^{-1}$ thiourea, 4% (*v*/*v*) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)) with vortex mixing. After incubation at 30 °C for 1 h, the solutions were clarified by centrifugation and the soluble proteins were used for two-dimensional electrophoresis analysis.

Protein samples (50 μg) were labeled with 400 pmol of Cy™ dye as specified by the manufacturer (GE Healthcare, Piscataway, New Jersey, USA). The ambient sample was labeled with Cy3 and the treated sample with Cy5. The internal standard was a pooled sample composed of equal amounts of each of the two samples that had been mixed and labeled with Cy2 dye. For two-dimensional analysis (2-DE), the first dimension was conducted with an Amersham Ettan™ IPGphor isoelectric focusing (IEF) system using mixtures of nontransgenic Col-0, plants overexpressing the 14-3-3ω$^{E208A}$ mutant, and internal standard protein. Total protein (500 μg) was actively loaded onto Immobiline™ DryStrip pH 3 to 11 (24 cm) strips and focused on the IPGphor IEF system for a total of 20 h (68 kVh) at 20 °C. After focusing, the strips were equilibrated for 10 min in 10 mL of equilibration buffer containing first DTT followed by buffer containing iodoacetamide. The equilibration buffer contained 50 mmol·L$^{-1}$ Tris-HCl, pH 8.8, 30% (*v*/*v*) glycerol, 2% (*w*/*v*) SDS, 6 mol·L$^{-1}$ urea, and 65 mmol·L$^{-1}$ DTT or 125 mmol·L$^{-1}$ iodoacetamide, as specified. The strips were then placed onto 12% SDS–PAGE gels and run for 6 h. Fluorescent gel images were obtained using a Typhoon™ 9400 multilaser scanner before post-staining with SyproRuby for total protein. Images were analyzed using the DeCyder Differential Analyzer software (GE Healthcare) and gel plugs were taken using the Ettan™ Spot Handling Workstation.

### In-gel trypsin digestion and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS) analysis

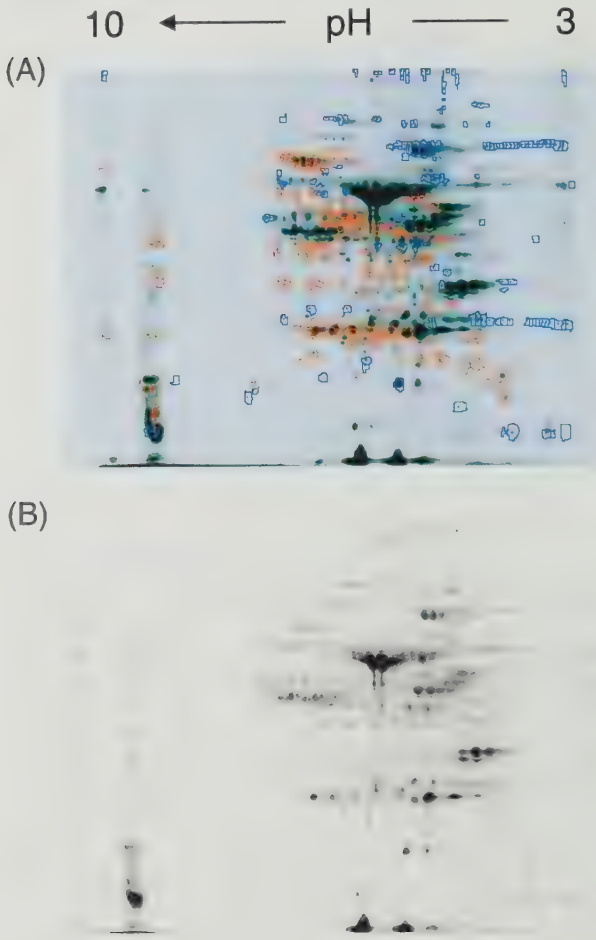
Gel plugs were rinsed with 50 mmol·L$^{-1}$ ammonium bicarbonate in 50% acetonitrile (ACN), and allowed to air dry. Modified trypsin (Genotech, St. Louise, Missouri, USA) was added to a final concentration of 0.0125 U·μL$^{-1}$ in 50 mmol·L$^{-1}$ ammonium bicarbonate to the gel plugs and allowed to digest for 4 h. The solution was then removed and an aliquot was mixed with saturated cyano-4-hydroxycinnamic acid (HCCA) in 50% (*v*/*v*) acetonitrile (CAN) containing 0.1% (*v*/*v*) trifluoroacetic acid (TFA). An aliquot (usually 0.3 μL) of the peptide–matrix mixture was spotted onto a GE Healthcare probe and allowed to air dry. MS analysis was performed with an Amersham Ettan™ MALDI-ToF/Pro spectrometer, and spectra were analyzed using Mascot to search the NCBInr database.

## Results

### Overexpression of 14-3-3ω affects nitrate reductase (NR) protein content

We transformed *Arabidopsis* plants to overexpress wild type 14-3-3ω or directed mutants (E208A or L212N) of loop 8 that affect the interaction with pNR. In vitro, the recombinant 14-3-3ω$^{E208A}$ mutant inhibited pNR activity significantly in the absence of $Mg^{2+}$ and produced even greater inhibition in the absence of $Mg^{2+}$ (Fig. 1), results consistent

**Fig. 7.** Two-dimensional differential in-gel electrophoresis (DIGE) analysis of total soluble protein from plants overexpressing 14-3-3ω$^{E208A}$ compared with nontransgenic *Arabidopsis* Col-0 plants. (A) DeCyder analysis identified 1100 distinct protein spots that were of similar abundance, and ~160 spots that were increased (blue spots) and ~200 spots that were decreased in abundance (red spots) in the E208A transgenic compared with nontransgenic wild type. (B) SyproRuby stained gel showing the protein abundance without annotations.




with an increased affinity for its client protein (Athwal and Huber 2002). We also constructed several other substitution mutants in the loop 8 of 14-3-3ω. Interestingly, the 14-3-3ω$^{L212N}$ mutant produced little or no inhibition of pNR activity either in the presence or absence of $Mg^{2+}$ at pH 7.5 (Fig. 1B), indicating a critical role for this hydrophobic residue in regulation of the enzymatic activity of the client. Together, these two directed mutations highlight the potential importance of loop 8 residues in interaction with the client protein. In contrast, alanine substitutions for three other acidic residues in loop 8 had little effect on inhibition of pNR activity. The D210A and D219A mutants were identical to wild type 14-3-3ω, whereas the E215A mutant was slightly less effective than wild type in inhibition of pNR activity (Fig. 1B). Previous studies with the D219A mutant indicated a slight reduction in inhibition of pNR (provide ref), which is generally consistent with the current results.

Under acidic pH values, 14-3-3s can inhibit pNR activity in the absence of $Mg^{2+}$ (Kaiser and Huber 1994) as a result of increased binding (Athwal et al. 2000), and this is clearly seen in the experiments conducted in the present study at pH 6.5 (Fig. 1B). What is particularly striking is that all of the directed mutants, with the exception of E215A, behaved identically to the wild type 14-3-3ω. Clearly the role of loop 8 residues in interactions with the client protein is different at pH 6.5 versus 7.5, as the E208A substitution that increased inhibition at pH 7.5 had no effect at pH 6.5, and the L212N substitution that prevented inhibition at pH 7.5 was fully functional at pH 6.5. Interaction at pH 6.5 may only be physiologically relevant under stress conditions, e.g., anoxia (Kaiser and Huber 2001).

The E208A mutant was unique in conferring at least partial independence of divalent cation regulation and also in increased apparent affinity for pNR and hence was selected for transformation studies. Likewise, the essentially inactive L212N mutant was also selected as a negative control. Overexpression of wild type 14-3-3ω and directed mutants in transgenic *Arabidopsis* was achieved and the level of expression in each line was quantified by immunoblotting with antibodies directed against a peptide sequence derived from the COOH-terminal polypeptide of 14-3-3ω. One aspect we wanted to explore was whether increased expression of the 14-3-3 proteins would affect the steady-state level of NR protein, because 14-3-3 binding can protect the client protein from proteolytic degradation (MacKintosh and Meek 2001; Tzivion and Avruch 2002). As shown in Fig. 2, increased expression of wild type 14-3-3 resulted in some increase in NR protein, but expression of 14-3-3ω$^{E208A}$ had a much larger effect, consistent with the notion that the E208A directed mutant had increased ability to interact with pNR. In contrast, overexpression of 14-3-3ω$^{L212N}$ did not result in increased levels of NR protein, which is also consistent with the dramatically reduced ability of this mutant to interact with pNR in vitro. These results also suggest that the $Mg^{2+}$-independent binding of 14-3-3s to pNR that can be observed at pH 6.5 in vitro (Fig. 1B) is not likely to play a role in vivo, at least when plants are not stressed, because at pH 6.5 the wild type 14-3-3ω and E208A and L212N mutants behaved similarly. Their differential ability to inhibit pNR activity was only evident at pH 7.5, which matches the effect observed on NR protein in vivo.

### Overexpression of 14-3-3ω$^{E208A}$ affects phosphorylation of nitrate reductase (NR) at the regulatory Ser-534 site

Since the 14-3-3ω$^{E208A}$ mutant has partial independence from $Mg^{2+}$ and appears to bind more strongly to pNR than does wild type 14-3-3ω, it was of interest to determine whether the light-dark regulation of NR phosphorylation in vivo was altered by expression of the 14-3-3 mutant protein. We monitored NR phosphorylation at the Ser-534 regulatory site using modification-specific antibodies. As shown in Fig. 3, these antibodies readily cross reacted with pNR extracted from dark leaves and the immunoblot signal was specifically eliminated by competition with the phosphopeptide used as antigen (pSer-534) but not the corresponding unphosphorylated peptide (Ser-534), demonstrating the specificity of

**Table 1.** Proteins altered in abundance in leaves over expressing 14-3-3ω (E208A) compared with wild type leaves.

| Protein | Spot No. | Gene locus | MALDI E-value | Coverage (%) | Ratio (E208A/WT) |
|---|---|---|---|---|---|
| Cyclin | 36 | At1g14750 | 0.015 | 17.4 | 1.12 |
| ATP synthase, α subunit | 102 | AtCg00120 | 0.001 | 17.2 | 1.19 |
| ATP synthase, α subunit | 126 | | 0.001 | 15.8 | 1.38 |
| ATP synthase, β subunit | 153 | AtCg00480 | 0.053 | 20.5 | 1.17 |
| Prolyl isomerase, ROC4 | 597 | At3g62030 | 0.041 | 28.5 | 1.20 |
| Prolyl isomerase, ROC4 | 574 | | 0.000 | 35.0 | 1.28 |
| β-glucosidase (BGLU18) | 10 spots (77 to 92) | At1g52400 | 0.000 to 0.049 (0.012±0.005) | 8.9 to 17.6 (13.1±1.0) | –1.66 to –2.15 (–1.97±0.28) |
| Carbonic anhydrase | 520 | At3g01500 | 0.017 | 15.6 | –1.71 |
| GST (GSTF9) | 6 spots (517 to 543) | At2g30860 | 0.000 to 0.034 (0.017±0.008) | 25.6 to 40.0 | –1.16 to –2.22 (–1.55±0.17) |
| GST (GSTF9) | 573 | | 0.000 | 40.0 | 1.15 |

**Note:** The E-value is the expectation score that the identification is incorrect and thus the lower the number the more confident is the identification.

the antibodies. We also produced antibodies directed against a sequence near the N-terminus of the protein so that we could monitor total NR protein level as well. As expected, the immunoblot signal obtained with the anti-NT antibodies was unaffected by the presence of the Ser-534 or pSer-534 peptides (Fig. 3). In an initial experiment, we harvested leaves in the morning after 2 h of illumination and then after transferring plants to the dark for 1 h. In the experiment presented in Fig. 4, nontransgenic plants were compared with three independent lines overexpressing 14-3-3$\omega^{E208A}$. The relative phosphorylation status of NR is expressed as the ratio of the immunoblot signals for anti-NR-pS534 compared with anti-NR-NT, to normalize the phospho-specific signal to total NR protein. As expected, the phosphorylation of Ser-534 was higher in the dark than in the light. In these experiments, total NR protein changed little between the light and dark samples and was 1.8-fold higher in the E208A transgenic lines compared with the nontransgenic leaves. Interestingly, the relative phosphorylation status of NR in plants expressing the E208A mutant was also higher than in nontransgenic plants in both light and dark samples, consistent with the notion of increased binding of 14-3-3$\omega^{E208A}$ to pNR in vivo. This effect was most pronounced in line B2601, where the difference between light and dark samples was almost completely eliminated. Increased phosphorylation of NR at the Ser-534 site, using the sequence-specific antibodies developed in this study, was confirmed with the use of commercially available phospho-specific antibodies that recognize a degenerated mode 1 14-3-3-binding site, [KR]-x-x-pS-x-P (Cell Signaling Technology, Danvers, MA, USA). As expected, these antibodies recognized the NR protein on immunoblots with a ~3-fold increase in signal in the E208A plants relative to the nontransgenic plants (data not shown).

As noted above, plants expressing the 14-3-3$\omega^{E208A}$ mutant protein consistently had higher levels of total NR protein compared with nontransgenic plants or plants expressing the wild type 14-3-3ω. To determine whether NR protein stability contributed to this difference, excised leaves were fed cycloheximide to block cytoplasmic protein synthesis and the loss of NR protein was followed with time. As shown in Fig. 5, the NR protein in leaves expressing 14-3-3$\omega^{E208A}$ had a half-life that was roughly two-fold higher compared with nontransgenic leaves. Thus, decreased degradation is at least one of the factors that could explain the increased NR protein levels, and would be consistent with the notion that 14-3-3 binding protects pNR from proteolysis.

### Overexpression of 14-3-3$\omega^{E208A}$ affects nitrate reductase (NR) activity

We monitored NR activity in the presence and absence of magnesium to assess activation state of the enzyme (Kaiser and Huber 1994; Glaab and Kaiser 1995) in relation to the relative phosphorylation of NR at the Ser-534 site in leaves harvested at different times of a day–night cycle. Results for plants expressing the 14-3-3$\omega^{E208A}$ mutant protein are compared with nontransgenic plants in Fig. 6. The relative phosphorylation status of Ser-534 was high at the end of the night cycle, decreased rapidly upon illumination and then increased again after 3 h of illumination. Thereafter, phosphorylation of Ser-534 remained relatively constant before increasing to the maximum level with the transition to darkness (Fig. 6A). The same profile was observed both for nontransgenic plants and the transgenic plants overexpressing the 14-3-3$\omega^{E208A}$ mutant protein, but in the latter case, the NR phosphorylation status was always maintained at a higher level. The activation state of NR is plotted in Fig. 6B. As shown, activation state was very low at the end of the night cycle, increased sharply upon illumination and then decreased again after several hours. In the nontransgenic plants, NR activation state was maintained at a relatively constant level until the end of the light period, when it decreased again to the lowest level. Generally similar results have been reported previously with *Arabidopsis* leaves (Gibon et al. 2004; Kulma et al. 2004). In contrast, leaves overexpressing the 14-3-3$\omega^{E208A}$ mutant protein had increased NR activation state early in the light period similar to wild type but NR activation state then continuously declined throughout the remainder of the day and was overall much lower during the remainder of the light period. Plotting NR activation state as a function of Ser-534 phosphorylation state yielded a statistically significant negative correlation ($R^2 = 0.529$, $p < 0.05$) (Fig. 6C).

### Overexpression of 14-3-3$\omega^{E208A}$ alters the abundance of other cellular proteins

Overexpression of the E208A mutant protein in *Arabidop-*

*sis* resulted in a much greater increase in NR protein content compared with overexpression of the wild type protein (Fig. 2), and thus we further characterized the leaf soluble proteome in plants overexpressing the E208A mutant protein to determine whether other proteins were also affected. A 2-DE DIGE analysis of proteins extracted from leaves of plants overexpressing the 14-3-3$\omega^{E208A}$ mutant protein compared with the nontransgenic control resolved more than 1100 discrete protein spots and identified ~160 spots that were increased in abundance and ~200 protein spots that were decreased in abundance in the transgenic plants (Fig. 7). A number of the proteins altered in abundance were identified by peptide mass fingerprinting. Because total soluble protein was used in this analysis, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) large and small subunits were major proteins in the samples and indeed a number of the protein spots that changed in abundance were minor protein spots derived from the large subunit (data not shown). However, a number of other proteins were found to be altered in abundance and some of those identified are listed in Table 1. Proteins that were increased in abundance included a cyclin, the α and β subunits of the plastid ATP synthase, and prolyl isomerase. Interestingly, of the proteins that were decreased in abundance were β-glucosidase (BGL1) and glutationine *S*-transferase (GST). Both proteins were found as charge trains composed of multiple spots, most of which were decreased in abundance in the E208A transgenic. The one exception was spot 573, identified as GST, that was increased slightly in abundance. It is important to note that while BGL1 and GSTF9 were identified, differentiation of various β-glucosidases and GST isoforms that may also be present in the charge train of protein spots was not possible by MALDI-ToF-MS analysis because of the high degree of sequence identity among isoforms. Moreover, previous studies with *Arabidopsis* GSTs resolved 23 protein spots in a large charge-train pattern on 2-DE, with individual isoforms found in multiple spots suggestive of post-translational modifications (Gruhler et al. 2005). Thus multiple GST isoforms were probably present in the gel plugs we sampled, and if so, most of them were reduced in abundance in plants overexpressing 14-3-3$\omega^{E208A}$ compared with nontransgenic plants. Likewise, the *Arabidopsis* Glycoside Hydrolase Family 1 β-glucosidases contains 47 members (Xu et al. 2004), and many are closely related in sequence and abundantly expressed in leaves. We identified BGLU18, also known as BGL1 (Stotz et al. 2000), but it is possible that other closely related isoforms were present as well, but not specifically identified. However, the important point is that the β-glucosidase isoforms present were primarily reduced in abundance in the E208A plants.

## Discussion

In the present study, we further characterized the role of specific residues in loop 8 of 14-3-3ω that impact binding to the client protein, pNR. Glu-208 is shown to be a unique acidic residue in loop 8 that when substituted with alanine results in a protein with substantial binding to pNR in the absence of magnesium. One of the hydrophobic residues of loop 8, Leu-212, is shown for the first time to be essential for regulation of pNR activity, which is consistent with the notion that loop 8 residues interact closely with the client protein (Sinnige et al. 2005; Wilker et al. 2005). We overexpressed several of these mutant 14-3-3 proteins in *Arabidopsis* to assess the impact on NR regulation and to see whether expression of mutant 14-3-3s might be an interesting approach to investigate the role of 14-3-3 proteins in vivo.

### Phosphorylation of nitrate reductase (NR) at the Ser-534 site controls activation state

There is substantial evidence to show that phosphorylation of NR at the Ser-534 site in hinge 1 completes the binding site for a 14-3-3 protein that interacts in the presence of magnesium to form an inactive pNR:14-3-3 complex (Bachmann et al. 1996*a*; MacKintosh and Meek 2001; Athwal and Huber 2002; Manak and Ferl 2007). The activation state of NR, calculated as the ratio of NR activities measured in the presence versus absence of magnesium, is frequently taken as a proxy measurement of the phosphorylation status of the regulatory serine residue. However, NR activity and Ser-534 phosphorylation need to be examined directly and independently to study this relationship in vivo and this has rarely been done. The simplest way to look at phosphorylation status of a specific site is through the use of sequence- and modification-specific antibodies. This was done in one study using antibodies produced against a synthetic peptide with an acidic residue as mimic for pSer-543 in spinach NR (Weiner and Kaiser 2001). The antibodies produced selectively recognized phospho- or dephospho-NR and were used to demonstrate that NR phosphorylation at Ser-543 increased 2 to 3-fold when spinach leaves were darkened. Interestingly, it was calculated that one-third of the NR in spinach leaves was phosphorylated in the light but was not associated with inactivation of the enzyme leading to the conclusion that phosphorylation of Ser-543 was not sufficient for 14-3-3 binding (Weiner and Kaiser 2001). Possible reasons for this could include lack of magnesium or other divalent cations required for 14-3-3 binding to pNR, or competition for 14-3-3 proteins with other clients. In *Arabidopsis* the situation is apparently somewhat different as changes in Ser-534 phosphorylation during a normal dark-to-light transition can be as high as seven-fold in nontransgenic plants (Fig. 6A). Moreover, relative phosphorylation of Ser-534 is negatively correlated with NR activation state (Fig. 6B) and the regression line extrapolates to ~85% activation state when phosphorylation is zero. Thus, at least in *Arabidopsis*, NR activation state is a good proxy measurement for the phosphorylation status of Ser-534. It is also possible that slightly different results were obtained in the present study because antibodies were prepared against the phosphoserine-containing peptide rather than an aspartate substitution mimic, which in the best case reacted rather poorly with pNR.

### The *Arabidopsis* pNR:14-3-3 complex is less susceptible to degradation

NR is generally considered to be a short-lived protein and there is often a substantial variation in NR protein content over a normal diurnal cycle. Changes in protein content reflect necessarily the balance between changes in synthesis and degradation of the protein, and both processes can be regulated. In the case of degradation, there is some contro-

versy in the literature as to whether the pNR:14-3-3 complex is more or less susceptible to proteolytic degradation compared with NR (or pNR) that is not bound to a 14-3-3 protein. For example, 14-3-3 binding to pNR has been suggested to promote proteolysis (Weiner and Kaiser 1999), inhibit proteolysis (Cotelle et al. 2000), or have no effect (Lillo et al. 2003). Previously, we noted that manipulation of NR activation state also affected half-life of the NR protein, with activation state and NR stability being positively correlated (Kaiser and Huber 2001). However, it was also noted that such correlations do not establish cause-and-effect relationships and that it remained to be determined whether NR degradation is affected by the NR form present in leaves and (or) by other factors that are co-regulated by conditions that impact NR activation (Kaiser and Huber 2001). An example of the latter would be induction or activation of a protease that degraded NR. The results obtained in the present study are consistent with the notion that the pNR:14-3-3 complex is protected from degradation, as overexpression of the 14-3-3$\omega^{E208A}$ mutant protein resulted in elevated NR protein levels. However, it is likely that control of protease induction/activation is also important and that both factors contribute to NR stability. This is also consistent with the results with sugar-starved *Arabidopsis* cells that were shown to induce a protease that cleaved proteins when not complexed with 14-3-3 proteins (Cotelle et al. 2000). However, it is important to note that while the generalization is often made that 14-3-3 binding can protect clients from degradation (Tzivion and Avruch 2002), there are other cases where the opposite may be true. In this regard, it was reported that binding of a 14-3-3 protein to phosphorylated glutamine synthetase (GS2) resulted in selective proteolysis of the protein (Man and Kaiser 2001; Lima et al. 2006). Conceivably, other proteins may respond similarly.

### The pNR:14-3-3 complex is less accessible to protein phosphatases

Since 14-3-3 proteins bind directly to the phosphoserine residue in the client protein, it would certainly be expected that access to action of a protein phosphatase would be restricted. This was originally demonstrated for Raf-1 (Muslin et al. 1996) and subsequently for pNR in vitro (Bachmann et al. 1996*a*) . The finding in the present study that overexpression of the 14-3-3$\omega^{E208A}$ mutant protein results in increased phosphorylation of the NR-Ser-534 site (Fig. 6A) is consistent with these in vitro results.

### Overexpression of 14-3-3$\omega^{E208A}$ affects the soluble leaf proteome

Because of the impact of E208A overexpression on NR protein, we wanted to determine whether other cellular proteins would be altered in abundance as well, as this could provide an in vivo approach to identify 14-3-3ω clients. Two-dimensional DIGE analysis of total soluble protein samples indicated that overexpression of the mutant 14-3-3$\omega^{E208A}$ affected the abundance of a number of proteins (Table 1), some of which are known to be 14-3-3 binding proteins. For example, binding of 14-3-3s to the phosphorylated β-subunit of plastid ATP synthase has been shown to occur and apparently functions to prevent ATP hydrolysis in the dark (Bunney et al. 2001). Likewise, GST was identified as a potential 14-3-3 binder in a yeast two-hybrid screen using a barley leaf cDNA library (Schoonheim et al. 2007). Collectively, these findings are generally consistent with the notion that many proteins involved in defense and stress responses are 14-3-3 binding proteins, as demonstrated for developing barley grains (Alexander and Morris 2006). Whether the basis for changes in abundance of proteins such as GSTF9 and BGLU18 identified in the present study involves direct binding of the proteins to 14-3-3$\omega^{E208A}$ is not known. Likewise, it is not clear whether changes in protein abundance reflect alterations in the rate of degradation or rate of synthesis, since many transcription factors are controlled by 14-3-3 proteins (Roberts 2003; Ferl 2004). In the future, it will be of interest to determine whether the reduced levels of these stress-related proteins impact the ability of the transgenic plants to deal with various stress conditions. The GSTs are multifunctional proteins, but in addition to their activity in xenobiotic detoxification, they can also function as glutathione peroxidases and thus can protect against oxidative stress (Roxas et al. 1997; Cummins et al. 1999; Roxas et al. 2000). Likewise, the β-glucosidases can potentially play many roles in processes related to lignification, release of hormones from glucose conjugates, and defense against herbivory (Xu et al. 2004). Indeed BGLU18 and several GSTs were induced in leaves in response to feeding of a specialist insect herbivore (Stotz et al. 2000), indicating their role as defense genes that contribute to induced resistance against herbivores and possibly other conditions as well. Exploring these aspects is beyond the scope of the present study but will be exciting to pursue in the future. It is also important to note that the DIGE analysis using total protein samples likely did not identify a number of low abundance proteins that may also have been altered in content. Thus, fractionation of samples to remove RubisCO and enrich low abundance proteins will likely be a useful strategy. Indeed, we have done this using ammonium sulfate fractionation and found a number of additional proteins that were increased or decreased in content in the transgenic plants (data not shown).

Because 14-3-3 proteins bind to a wide variety of cellular target proteins, manipulation of 14-3-3s (by overexpression or use of knockout lines) is viewed as a possible approach to influence entire pathways or processes. Previously, manipulation of 14-3-3s has been shown to influence starch accumulation (Sehnke et al. 2001), metabolite profiles (Szopa 2002), and leaf senescence (Yan et al. 2004). Our results suggest that overexpressing 14-3-3 mutants may also be an interesting approach to investigate 14-3-3 function in vivo.

## Acknowledgements

This research was supported by the United States Department of Agriculture (USDA) - National Research Initiative (NRI) (Agreement No. 2007-35318-17801) and USDA, Agricultural Research Service (ARS). Any opinions, findings, and conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the USDA-ARS or USDA-NRI. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the

USDA. We thank Dan Person for performing the experiments reported in Fig. 5.

## References


Alexander, R.D., and Morris, P.C. 2006. A proteomic analysis of 14-3-3 binding proteins from developing barley grains. Proteomics, **6**: 1886–1896. doi:10.1002/pmic.200500548. PMID:16470656.

Athwal, G.S., and Huber, S.C. 2002. Divalent cations and polyamines bind to loop 8 of 14-3-3 proteins, modulating their interaction with phosphorylated nitrate reductase. Plant J. **29**: 119–129. doi:10.1046/j.0960-7412.2001.01200.x. PMID:11851916.

Athwal, G.S., Lombardo, C., Huber, J.L., Masters, S.C., Fu, H., and Huber, S.C. 2000. Modulation of 14-3-3 protein interactions with target polypeptides by physical and metabolic effectors. Plant Cell Physiol. **41**: 523–533. PMID:10845467.

Bachmann, M., Huber, J.L., Athwal, G.S., Wu, K., Ferl, R.J., and Huber, S.C. 1996*a*. 14-3-3 proteins associate with the regulatory phosphorylation site of spinach leaf nitrate reductase in an isoform-specific manner and reduce dephosphorylation by endogenous protein phosphatases. FEBS Lett. **398**: 26–30. doi:10.1016/S0014-5793(96)01188-X. PMID:8946947.

Bachmann, M., Huber, J.L., Liao, P.-C., Gage, D.A., and Huber, S.C. 1996*b*. The inhibitor protein of phosphorylated nitrate reductase from spinach (*Spinacia oleracea*) leaves is a 14-3-3 protein. FEBS Lett. **387**: 127–131. doi:10.1016/0014-5793(96)00478-4. PMID:8674533.

Bachmann, M., Shiraishi, N., Campbell, W.H., Yoo, B.-C., Harmon, A.C., and Huber, S.C. 1996*c*. Identification of Ser-543 as the major regulatory phosphorylation site in spinach leaf nitrate reductase. Plant Cell, **8**: 505–517. doi:10.1105/tpc.8.3.505. PMID:8721752.

Bunney, T.D., van Walraven, H.S., and de Boer, A.H. 2001. 14-3-3 protein is a regulator of the mitochondrial and chloroplast ATP synthase. Proc. Natl. Acad. Sci. U.S.A. **98**: 4249–4254. doi:10.1073/pnas.061437498. PMID:11274449.

Clough, S.J., and Bent, A.F. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. **16**: 735–743. doi:10.1046/j.1365-313x.1998.00343.x. PMID:10069079.

Cotelle, V., Meek, S.E.M., Provan, F., Milne, F.C., Morrice, N., and MacKintosh, C. 2000. 14-3-3s regulate global cleavage of their diverse binding partners in sugar-starved *Arabidopsis* cells. EMBO J. **19**: 2869–2876. doi:10.1093/emboj/19.12.2869. PMID:10856232.

Cummins, I., Cole, D.J., and Edwards, R. 1999. A role for glutathione transferases functioning as glutathione peroxidases in resistance to multiple herbicides in black-grass. Plant J. **18**: 285–292. doi:10.1046/j.1365-313X.1999.00452.x. PMID:10377994.

Douglas, P., Morrice, N., and MacKintosh, C. 1995. Identification of a regulatory phosphorylation site in the hinge 1 region of nitrate reductase from spinach (*Spinacea oleracea*) leaves. FEBS Lett. **377**: 113–117. doi:10.1016/0014-5793(95)01300-8. PMID:8543031.

Ferl, R.J. 2004. 14-3-3 proteins: regulation of signal-induced events. Physiol. Plant. **120**: 173–178. doi:10.1111/j.0031-9317.2004.0239.x. PMID:15032850.

Gibon, Y., Blaesing, O.E., Hannemann, J., Carillo, P., Höhne, M., Hendriks, J.H.M., Palacios, N., Cross, J., Selbig, J., and Stitt, M. 2004. A robot-based platform to measure multiple enzyme activities in *Arabidopsis* using a set of cycling assays: comparison of changes of enzyme activities and transcript levels during diurnal cycles and in prolonged darkness. Plant Cell, **16**: 3304–3325. doi:10.1105/tpc.104.025973. PMID:15548738.

Glaab, J., and Kaiser, W.M. 1995. Inactivation of nitrate reductase involves NR-protein phosphorylation and subsequent 'binding' of an inhibitor protein. Planta, **195**: 514–518. doi:10.1007/BF00195708.

Glaab, J., and Kaiser, W.M. 1996. The protein kinase, protein phosphatase and inhibitor protein of nitrate reductase are ubiquitous in higher plants and independent of nitrate reductase expression and turnover. Planta, **199**: 57–63. doi:10.1007/BF00196881.

Gruhler, A., Schulze, W.X., Matthiesen, R., Mann, M., and Jensen, O.N. 2005. Stable isotope labeling of *Arabidopsis thaliana* cells and quantitative proteomics by mass spectrometry. Mol. Cell. Proteomics, **4**: 1697–1709. doi:10.1074/mcp.M500190-MCP200. PMID:16088002.

Hoagland, D. R., and Arnon, D. I. 1950. Calif Agric Exp Stn Bull, Circular 347.

Kaiser, W.M., and Huber, S.C. 1994. Modulation of nitrate reductase in vivo and in vitro: effects of phosphoprotein phosphatase inhibitors, free $Mg^{2+}$ and 5′-AMP. Planta, **193**: 358–364. doi:10.1007/BF00201813.

Kaiser, W.M., and Huber, S.C. 2001. Post-translational regulation of nitrate reductase: mechanism, physiological relevance and environmental triggers. J. Exp. Bot. **52**: 1981–1989. doi:10.1093/jexbot/52.363.1981. PMID:11559733.

Kulma, A., Villadsen, D., Campbell, D.G., Meek, S.E.M., Harthill, J.E., Nielsen, T.M., and MacKintosh, C. 2004. Phosphorylation and 14-3-3 binding of *Arabidopsis* 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. Plant J. **37**: 654–667. doi:10.1111/j.1365-313X.2003.01992.x. PMID:14871307.

Lillo, C., Lea, U.S., Leydecker, M.-T., and Meyer, C. 2003. Mutation of the regulatory phosphorylation site of tobacco nitrate reductase results in constitutive activation of the enzyme in vivo and nitrite accumulation. Plant J. **35**: 566–573. doi:10.1046/j.1365-313X.2003.01828.x. PMID:12940950.

Lima, L., Seabra, A., Melo, P., Cullimore, J., and Carvalho, H. 2006. Phosphorylation and subsequent interaction with 14-3-3 proteins regulate plastid glutamine synthetase in *Medicago truncatula*. Planta, **223**: 558–567. doi:10.1007/s00425-005-0097-8. PMID:16136328.

Lu, G., Sehnke, P.C., and Ferl, R.J. 1994. Phosphorylation and calcium binding properties of an *Arabidopsis* GF14 brain protein homolog. Plant Cell, **6**: 501–510. doi:10.1105/tpc.6.4.501. PMID:8205002.

MacKintosh, C. 2004. Dynamic interactions between 14-3-3 proteins and phosphoproteins regulate diverse cellular processes. Biochem. J. **381**: 329–342. doi:10.1042/BJ20031332. PMID:15167810.

MacKintosh, C., and Meek, S.E.M. 2001. Regulation of NR activity by reversible phosphorylation, 14-3-3 proteins and proteolysis. Cell. Mol. Life Sci. **58**: 205–214. doi:10.1007/PL00000848. PMID:11289302.

Man, H.-M., and Kaiser, W.M. 2001. Increased glutamine synthetase activity and changes in amino acid pools in leaves treated with 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR). Physiol. Plant. **111**: 291–296. doi:10.1034/j.1399-3054.2001.1110305.x. PMID:11240912.

Manak, M.S., and Ferl, R.J. 2007. Divalent cation effects on interactions between multiple *Arabidopsis* 14-3-3 isoforms and phosphopeptide targets. Biochemistry, **46**: 1055–1063. doi:10.1021/bi061366c. PMID:17240989.

Moorhead, G., Douglas, P., Morrice, N., Scarabel, M., Aitken, A., and MacKintosh, C. 1996. Phosphorylated nitrate reductase from spinach leaves is inhibited by 14-3-3 proteins and activated by

fusicoccin. Curr. Biol. **6**: 1104–1113. doi:10.1016/S0960-9822(02)70677-5. PMID:8805370.

Moorhead, G., Douglas, P., Cotelle, V., Harthill, J., Morrice, N., Meek, S., Deiting, U., Stitt, M., Scarabel, M., Aitken, A., and MacKintosh, C. 1999. Phosphorylation-dependent interactions between enzymes of plant metabolism and 14-3-3 proteins. Plant J. **18**: 1–12. doi:10.1046/j.1365-313X.1999.00417.x. PMID: 10341439.

Muslin, A.J., Tanner, J.W., Allen, P.M., and Shaw, A.S. 1996. Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. Cell, **84**: 889–897. doi:10.1016/S0092-8674(00)81067-3. PMID:8601312.

Roberts, M.R. 2003. 14-3-3 proteins find new partners in plant cell signaling. Trends Plant Sci. **8**: 218–223. doi:10.1016/S1360-1385(03)00056-6. PMID:12758039.

Roxas, V.P., Smith, R.K.J., Allen, E.R., and Allen, R.D. 1997. Overexpression of glutathione *S*-transferase/glutathione peroxidase enhances the growth of transgenic tobacco seedlings during stress. Nat. Biotechnol. **15**: 988–991. doi:10.1038/nbt1097-988. PMID:9335051.

Roxas, V.P., Lodhi, S.A., Garrett, D.K., Mahan, J.R., and Allen, R.D. 2000. Stress tolerance in transgenic tobacco seedlings that overexpress glutathione *S*-transferase/glutathione peroxidase. Plant Cell Physiol. **41**: 1229–1234. doi:10.1093/pcp/pcd051. PMID:11092907.

Schoonheim, P.J., Veiga, H., da Costa Pereira, D., Friso, G., van Wijk, K.J., and de Boer, A.H. 2007. A comprehensive analysis of the 14-3-3 interactome in barley leaves using a complementary proteomics and two-hybrid approach. Plant Physiol. **143**: 670–683. doi:10.1104/pp.106.090159. PMID:17172288.

Sehnke, P.C., Chung, H.-J., Wu, K., and Ferl, R.J. 2001. Regulation of starch accumulation by granule-associated plant 14-3-3 proteins. Proc. Natl. Acad. Sci. U.S.A. **98**: 765–770. doi:10.1073/pnas.021304198. PMID:11149942.

Shen, W., and Huber, S.C. 2006. Polycations globally enhance binding of 14-3-3ω to target proteins in spinach leaves. Plant Cell Physiol. **47**: 764–771. doi:10.1093/pcp/pcj050. PMID:16621845.

Shen, W., Clark, A.C., and Huber, S.C. 2003. The C-terminal tail of *Arabidopsis* 14-3-3 omega functions as an autoinhibitor and may contain a tenth alpha-helix. Plant J. **34**: 473–484. doi:10.1046/j.1365-313X.2003.01739.x. PMID:12753586.

Sinnige, M.P., Roobeek, I., Bunney, T.D., Visser, A.J.W.G., Mol, J.N.M., and de Boer, A.H. 2005. Single amino acid variation in barley 14-3-3 proteins leads to functional isoform specificity in the regulation of nitrate reductase. Plant J. **44**: 1001–1009. doi:10.1111/j.1365-313X.2005.02599.x. PMID:16359392.

Stotz, H.U., Pittendrigh, B.R., Kroymann, J., Weniger, K., Fritsche, J., Bauke, A., and Mitchell-Olds, T. 2000. Induced plant defense responses against chewing insects. Ethylene signaling reduces resistance of *Arabidopsis* against Egyptian cotton worm but not diamondback moth. Plant Physiol. **124**: 1007–1017. doi:10.1104/pp.124.3.1007. PMID:11080278.

Su, W., Huber, S.C., and Crawford, N.M. 1996. Identification in vitro of a post-translational regulatory site in the hinge 1 region of *Arabidopsis* nitrate reductase. Plant Cell, **8**: 519–527. doi:10.1105/tpc.8.3.519. PMID:8721753.

Szopa, J. 2002. Transgenic 14-3-3 isoforms in plants: the metabolite profiling of repressed 14-3-3 protein synthesis in transgenic potato plants. Biochem. Soc. Trans. **30**: 405–410. doi:10.1042/BST0300405. PMID:12196104.

Tzivion, G., and Avruch, J. 2002. 14-3-3 proteins: active cofactors in cellular regulation by serine/threonine phosphorylation. J. Biol. Chem. **277**: 3061–3064. doi:10.1074/jbc.R100059200. PMID:11709560.

Weiner, H.K., and Kaiser, W.M. 1999. 14-3-3 proteins control proteolysis of nitrate reductase in spinach leaves. FEBS Lett. **455**: 75–78. doi:10.1016/S0014-5793(99)00868-6. PMID:10428475.

Weiner, H., and Kaiser, W.M. 2001. Antibodies to assess phosphorylation of spinach leaf nitrate reductase on serine 543 and its binding to 14-3-3 proteins. J. Exp. Bot. **52**: 1165–1172. doi:10.1093/jexbot/52.359.1165. PMID:11432934.

Wilker, E.W., Grant, R.A., Artim, S.C., and Yaffe, M.B. 2005. A structural basis for 14-3-3σ functional specificity. J. Biol. Chem. **280**: 18891–18898. doi:10.1074/jbc.M500982200. PMID: 15731107.

Xu, Z., Escamilla-Treviño, L.L., Zeng, L., Lalgondar, L., Bevan, D.R., Winkel, B.S.J., Mohamed, A., Cheng, C.-L., Shih, M.-C., Poulton, J.E., and Esen, A. 2004. Functional genomic analysis of *Arabidopsis thaliana* glycoside hydrolase family 1. Plant Mol. Biol. **55**: 343–367. doi:10.1007/s11103-004-0790-1. PMID: 15604686.

Yan, J., He, C., Wang, J., Mao, Z., Holaday, S.A., Allen, R.D., and Zhang, H. 2004. Overexpression of the *Arabidopsis* 14-3-3 protein GF14 lambda in cotton leads to a "stay-green" phenotype and improves stress tolerance under moderate drought conditions. Plant Cell Physiol. **45**: 1007–1014. doi:10.1093/pcp/pch115. PMID:15356326.

# Site- and species-specific patterns of metal bioavailability in edible plants[1]

**Hollydawn Murray, Karen Thompson, and Sheila M. Macfie**

**Abstract:** Differences in metal uptake between plant species and soil types were compared to assess the safe use of mildly contaminated soils for the growth of edible food crops. Accumulation of metals in five plant species grown in each of three field soils and a commercial soil were evaluated in a controlled environment. Metal bioavailability varied more with plant species than with type of soil. Among a number of physical and chemical soil properties that were measured, high metal content and low percent organic matter were the best predictors of increased metal bioavailability. Contamination levels of metals measured in soil and vegetable samples were used to calculate bioconcentration factors and hazard quotients. The results indicated significant differences between plant species. The species accumulating the most metal was carrot, and the most mobile element was cadmium. Some hazard quotients exceeded the threshold value of 1, even in soils considered uncontaminated by current guidelines. Overall, these results reinforce the need to include soil characteristics when setting threshold guidelines for metal content of agricultural soils and indicate the need for species-specific planting guidelines.

*Key words:* cadmium, copper, lead, zinc, plant uptake, risk assessment, hazard quotient.

**Résumé :** Les auteurs ont comparé les différences dans l'absorption des métaux entre des espèces de plantes et des types de sols pour évaluer l'utilisation sécuritaire des sols modérément contaminés pour la production de denrées comestibles. Les auteurs ont évalué, en chambres à environnement contrôlé, l'accumulation des métaux chez cinq espèces de plantes chacune dans trois sols prélevés aux champs. La biodisponibilité des métaux varie plus fortement avec les espèces de plantes qu'avec les types de sols. Parmi un ensemble de propriétés physiques et chimiques du sol déterminées, la teneur en métaux et le faible contenu en matière organique constituent les meilleurs indicateurs de l'augmentation de la biodisponibilité des métaux. Les auteurs ont utilisé les degrés de contamination en métaux mesurés dans les échantillons des sols et des légumes pour calculer les facteurs de bioconcentration et les quotients de risque. Les résultats montrent des différences significatives entre les espèces de plantes. La carotte est celle qui accumule le plus de métaux et le cadmium représente l'élément le plus mobile. Quelques quotients de risque dépassent la valeur seuil, même dans des sols non contaminés selon les guides actuels. Dans l'ensemble, ces résultats renforcent le besoin d'inclure les caractéristiques des sols lorsqu'on établit des guides de seuils critiques pour les teneurs en métaux des sols agricoles et soulignent le besoin d'établir des guides spécifiques à l'espèce cultivée.

*Mots-clés :* cadmium, cuivre, plomb, zinc, absorption par les plantes, évaluation du risque, quotient de risque.

[Traduit par la Rédaction]

## Introduction

### Metal contamination in soils

Some metals (e.g., Cu, Zn, Mn) are required, at low concentrations, in plants for structurally catalytic components of proteins and enzymes; however, high concentrations of both essential and nonessential metals can be toxic (Van Aasche and Clijsters 1990). Elevated amounts of metals in soils arise from natural ore bodies, and from anthropogenic sources such as industrial production, mining, sewage sludge usage, and fertilizer application (reviewed in Ross 1994). Such contamination can affect humans directly through inadvertent consumption of soil or, more commonly, ingestion of crops grown under contaminated conditions. For example, elevated concentrations of Cd in soils used to grow rice (*Oryza sativa* L.) led to human renal tubular dysfunction among Asian families (Kasuya 2000).

### Soil metal regulation

Recognizing the need to reduce risk to human consumers, several countries have established limits to the permissible concentrations for many potentially toxic metals in agricultural soils. These agricultural threshold limits, including those set by the Canadian Council of the Ministers of the Environment (CCME 2006), are based on the concentration of metals in the bulk soil (total metal). However, some metal ions are tightly bound within the soil and only a fraction of the total metal in a soil is bioaccessible (available for

Received 30 October 2008. Published on the NRC Research Press Web site at botany.nrc.ca on 15 July 2009.

**H. Murray, K. Thompson,[2] and S.M. Macfie.[3]** Department of Biology, University of Western Ontario, London, ON N6A 5B7, Canada.

[1]This paper is one of a selection published in a Special Issue comprising papers presented at the 50th Annual Meeting of the Canadian Society of Plant Physiologists (CSPP) held at the University of Ottawa, Ontario, in June 2008.

[2]Present address: The Language Education Institute of Chosun University, 375 Seosuk-dong, Dong-gu, Gwangju, 501-759, South Korea.

[3]Corresponding author (e-mail: smacfie@uwo.ca).

uptake), and only a portion of the bioaccessible pool is bioavailable (actually taken up) (Peijnenburg and Jager 2003). Because of this, current limits to metal content in agricultural soil may overestimate the potential for elevated concentrations of metals in plants (Rieuwerts et al. 1998). There is a general consensus that total soil metal content is not an adequate predictor of metal bioavailability, and is not useful in assessing potential risk from soil contamination (Tack and Verloo 1995; Sauvé et al. 1999, 2000).

While the mechanisms of metal uptake are not entirely clear, it is believed that metals are taken up in their ionic form via active and passive transport (Clemens et al. 2002). Adsorption processes in the soil largely control the release of metal ions into soil solution, hence they influence the bioaccessibility of metals to plants (Bradl 2004). Thus, factors influencing metal adsorption and distribution regulate the availability of metals to plants (reviewed in McBride 1989). Fine textured soils tend to decrease metal bioaccessibility, owing to increased adsorption of metals to exchange sites on clay colloids (Brazauskiene et al. 2008). Metal solubility generally increases as soil pH decreases (Evans et al. 1995), and both conductivity and cation exchange capacity are positively correlated with metal bioaccessibility (Shuman 1999). These physicochemical factors, which greatly affect the bioaccessibility of metals, should be factored into the development of more realistic agricultural threshold limits (Jung and Thornton 1996). Because thorough physical and chemical analyses are costly and time-consuming, there is a need for risk assessment models that use a reduced set of physical and chemical variables to predict metal uptake by plants.

## Screening level risk assessment

At the screening level, the potential for a substance to present a health hazard can be estimated by assessing the amounts of known hazards in that substance. A number of approaches have been taken to assess the risk to humans of eating food produced on contaminated soils. One such method is the calculation of a biotic concentration factor (BCF), which is the ratio of the concentration of metal accumulated in the plant to the concentration of the metal in the soil (reviewed in Antunes et al. 2006). An index of the translocation of metal from root to shoot can be calculated by dividing the BCF for aboveground tissue by the BCF for root tissue. Plants (or edible tissues) with a higher BCF present a greater potential hazard to consumers.

Alternatively, one can compare the absolute amount of a potential hazard in food to the amount of that hazard that is known to cause adverse health effects. The Codex Alimentarius Commission (CAC), which is a joint Commission of the World Health Organization and the Food and Agriculture Organization, has established international standards for both organic and inorganic contaminants in food and animal feed (CAC 2007). The CAC used a combination of scientific information (e.g., toxicological data, estimates of typical consumptions patterns, and identification of susceptible groups) and political considerations (e.g., fair trade, barriers to international trade, and technological or processing limitations) to establish a "Codex limit" for many pesticides and metals. Food that contains concentrations higher than the Codex limit are not permitted on the international market (CAC 2007).

Another approach to screening for a potential risk of consumption involves the calculation of a "hazard quotient" (HQ; Pierzynski et al. 2000), which is a measure of the amount of a metal consumed relative to the threshold of toxicity of that metal for humans. If the HQ is below 1, there is no potential discernable risk to consuming the food; if the HQ is above 1, then the consumer is potentially at risk of exposure to toxic levels of metal contaminants. Sipter et al. (2008) used HQ to assess the risk of consuming vegetables grown in contaminated gardens in Hungary. Although the concentrations of As, Cd, and Zn exceeded the limits for agricultural soil, the corresponding HQs were below 0.05, indicating that none of the six vegetables tested accumulated metals to a potentially dangerous level.

## Influence of plant species and plant tissue on metal bioavailability

Current models of risk assessment and tolerance thresholds for agricultural soils focus on metal-specific guidelines; however, two plant species grown in the same soil do not necessarily pose the same risk to human consumers. Different plant species have different abilities to accumulate metal ions, both overall and within specific tissue types (Alexander et al. 2006). For example, spinach (*Spinacia oleracea* L.) leaves have higher concentrations of Mn than do spinach roots, yet within radish (*Raphanus sativus* L.) the highest concentrations of Mn are found in the roots (Intawongse and Dean 2006). Moreover, plant leaves often have higher metal concentrations than stems; thus, leafy vegetables grown on contaminated soils may pose the greatest risk (Qadir et al. 2000; Harrison 2001). Cultivars within a species may also vary in metal uptake (Ingwersen and Streck 2005). In addition, different metals have varying bioavailability. For example, in vegetables grown in metal-contaminated soil, Mn and Zn were readily bioavailable whereas Cu, Cd, and Pb were less bioavailable (Intawongse and Dean 2006).

A number of physiological variables influence the amount of metal taken up and translocated within a plant (reviewed in Clemens et al. 2002). To date, studies of the movement of metals from soil to plants have not revealed any predictable patterns and have varied with respect to the metal in question, the plant species used, or the plant tissue tested (Sinha et al. 2006). Because it is difficult to generalize patterns of metal uptake and accumulation from one species, or cultivar, to another, and different life stages of a single species may behave differently (Page et al. 2006), it understandable that models of risk assessment do not take plant species into consideration. However, as more species-specific information is gathered, it may be possible to place crops into a number of categories and subsequently establish planting guidelines that better reflect the interaction between soil, type of crop and metal accumulation.

## Objectives

In this study, the bioavailability of metals for five edible food crops were determined to assess the need for species-specific planting guidelines for soils with low to moderate levels of metal contamination. For four metals (Cd, Pb, Zn, and Cu), the potential health risks to humans consuming the vegetables were estimated.

## Materials and methods

### Soil preparation

Soils were obtained from the topsoil layer (25 cm) of an agricultural site (A) and a forested site (F) near Salaberry-de-Valleyfield, Quebec (45°16′N, 74°06′W). A third soil (L) was obtained from an agricultural site northeast of London, Ontario (43°7′N, 81°26′W). These three sites were chosen because they have elevated concentrations of a number of metals, including Cd and Pb in soils A and F (Ge and Hendershot 2005) and Cd in soil L (Shute and Macfie 2006). Soil F was not meant to represent a soil in which crops would normally be grown, it was chosen to increase the range of concentrations of metals in the experimental soils. We collected only one soil sample from each location because we were more interested in having four distinct soil types than in trying to characterize or represent the soil heterogeneity within each field site. A commercial potting soil, Promix (P), was used for comparative purposes. All soil samples were air-dried and sieved to ≤2 mm as per guidelines set out by the Metals in the Human Environment-Strategic Network (MITHE-SN) (MITHE-SN 2007).

### Metal content of the soil

Acid digestion for metal content followed the United States Environmental Protection Agency (US EPA) test method SW-846 (US EPA 2005). This procedure extracts all metals except those tightly bound in silicate minerals, which are not bioaccessible under natural conditions. For three replicates of each soil, 1.0 g soil was digested in 1 mL OmniTrace® nitric acid (EMD Chemicals Inc., Gibbstown, New Jersey, USA) in a glass test tube capped with a glass marble. Samples were digested at 100 °C until the vapours were clear (approx. 3 h). After cooling to room temperature, samples were filtered (qualitative paper No. 413, VWR International, Mississauga, Ontario, Canada) then brought to a final volume of 50 mL with distilled water. Controls of distilled water, $HNO_3$, and Montana Soil (Standard Reference Material 2711, National Institute of Standards and Technology, Gaithersburg, Maryland, USA) were similarly processed and analyzed. Digested samples were stored at 4 °C until analyzed using inductively coupled plasma – atomic emission spectroscopy (ICP-AES; Perkin Elmer Optima-3300 DV ICP-AES: RF generator power, 1300 W; gas flow rate, 15 L·min$^{-1}$; auxillary flow rate, 0.5 L·min$^{-1}$). The detection limit for each element was calculated to be equal to three times the standard deviation of readings for the nitric acid blanks.

### Soil characterization

All soils were analyzed in triplicate for general soil properties. Soil conductivity was measured after equilibration for 30 min in deionized water at a 10:1 liquid–soil ratio with a Hanna Instruments 8033 electrode (Hanna Instruments, Laval, Quebec, Canada) calibrated at 25 °C and 1413 μS·cm$^{-1}$. The pH of the supernatants from these solutions was then measured using a glass electrode calibrated at pH 4 and 10. Soil texture was determined using a sedimentation method adapted from Diaz-Zorita et al. (2002), which involved physical fractionation of sand, silt, and clay particles suspended in 1% sodium pyrophosphate. Organic matter content was measured via loss on ignition at 500 °C for 12 h (Heiri et al. 2001).

### Pot trials

Five crop species: carrot (*Daucus carota* L.); radish (*Raphanus sativus* L.); lettuce (*Lactuca sativa* L.); soybean (*Glycine max* (L.) Merr.); and wheat (*Triticum aestivum* L.), were potted in each of the four sieved soils, one plant per 10 cm diameter pot, and placed in a greenhouse with natural lighting, supplemented by halide lamps on a 16 h photoperiod at a minimum temperature of 25 ± 2 °C. Pots were watered daily to 75% field capacity with deionized water. Six millilitres of Hoagland's nutrient solution (Hoagland and Arnon 1950) was administered to all of the emerged seedlings twice a week for the duration of their growth period. Upon maturation of the edible tissues (approx. days to maturity: carrot, 65; radish, 28; lettuce, 45; soybean, 80; wheat, 65), plants were harvested. Roots of carrot and radish were rinsed in distilled water for 5 min, followed by a wash in 1 mmol·L$^{-1}$ $CaSO_4 \cdot 2H_20$ for 30 min to remove metals adsorbed to the root surface (Taylor et al. 1998), followed by a rinse in distilled water for 5 min. Lettuce leaves were rinsed in deionized water and blotted dry. Wheat grain and soybeans were untreated. All plant tissues were oven-dried to a constant mass at 60 °C.

### Plant tissue analysis

Edible tissues were analyzed for total metal content following US EPA test method SW-846 (US EPA 2005) in the same manner as for soil samples, with 0.1 g dry tissue being digested in 1 mL OmniTrace® nitric acid. Controls of distilled water, $HNO_3$, and tomato leaves (Standard Reference Material 1573a, National Institute of Standards and Technology) were similarly processed and analyzed. The metal content of digested plant tissues was determined using ICP-AES, as described above for soil.

To compare the amounts of Cd, Pb, Zn, and Cu accumulated by the edible tissue across soil types, bioconcentration factors (BCF) were calculated using eq. 1 (modified from Antunes et al. 2006):

$$[1] \quad BCF = M_e / M_s$$

Where $M_e$ = mean concentration of the metal in the edible tissue (mg·kg$^{-1}$ dry mass) and $M_s$ = mean concentration of the metal in the soil (mg·kg$^{-1}$ dry soil).

### Statistical analysis

Soil metal content and general soil properties were analyzed by one-way ANOVA. For all ANOVA where significant ($p < 0.05$) main effects were detected, Tukey's test of multiple comparisons was applied. Plant tissue metal concentrations were compared among species and between soils by means of multiple analyses of variance (MANOVA). Normality and correlation tests were undertaken prior to data testing. All statistics were performed using SAS Version 16.0.

### Screening level risk assessment

In terms of risk to human health, ingestion of vegetables grown under contaminated conditions was the only exposure pathway considered in this study. The average daily dose

(ADD) was calculated using eq. 2 (modified from Sipter et al. 2008):

$$[2] \quad ADD = (C_{veg} \times Y_{veg})/BW$$

Where $C_{veg}$ = concentration of the metal in the fresh edible tissue (mg·kg$^{-1}$ fresh mass), $Y_{veg}$ = average amount of the vegetable consumed (kg·d$^{-1}$), and BW = average bodyweight (kg). The ADD was calculated separately for Cd, Pb, Zn, and Cu, as well as for males and females, owing to differences in average bodyweight (69.4 kg for females, 82.6 kg for males, Sipter et al. 2008). Average consumption values were obtained from Canada's Food Guide (Health Canada 2008).

The noncarcinogenic risk of vegetable consumption was characterized using a hazard quotient (HQ), which is the ratio of the ADD to the reference dose (RfD) for each metal (Pierzynski et al. 2000). A reference dose corresponds to the maximum amount of a metal that can safely be consumed per day, per kilogram of bodyweight. Reference doses were obtained from the Integrated Risk Information System (US EPA 2006): 0.001 mg·kg$^{-1}$·d$^{-1}$ for Cd; 0.035 mg·kg$^{-1}$·d$^{-1}$ for Pb; 0.3 mg·kg$^{-1}$·d$^{-1}$ for Zn; and 0.04 mg·kg$^{-1}$·d$^{-1}$ for Cu. If HQ is greater than 1, then the ADD of that particular metal exceeds the RfD, indicating that there is a potential risk associated with consumption of food containing that metal.

## Results

### Soil characterization

The commercial potting soil (P) and the agricultural soil from London (L) had a higher pH than the two soils from Quebec (A and F) (Table 1). Soil P, which contains peat, had 2.5–4 times more organic matter when compared with the three field soils (Table 1). According to the United States Department of Agriculture Soil Texture Triangle (USDA 2008), which is based on the percentages of sand, silt, and clay, soils P, L, A, and F are classified as sandy loam, clay loam, silt loam, and loamy sand, respectively. Conductivity in the four soils ranged from 83 µS·cm$^{-1}$ in A to 397 µS·cm$^{-1}$ in P (Table 1).

Concentrations of Ba, Co, Cr, Mo, Ni, and Se did not differ among the four types of soil ($p$ = 0.238 to 0.843, data not shown). The concentrations of these six elements were below the Canadian Soil Quality Guidelines established by the Canadian Council of Ministers of the Environment (in mg·kg$^{-1}$; Ba 750; Co 40; Cr 64; Mo 5; Ni 50; Se 1;CCME 2006). The concentration (mean ± SE) of Al was highest in soil F (38.2 ± 0.4 × 10$^3$ mg·kg$^{-1}$), and lowest in soil P (15.7 ± 0.4 × 10$^3$ mg·kg$^{-1}$; $p < 0.001$). Soil F contained the highest concentration of Mn (1068 ± 26 mg·kg$^{-1}$); the lowest Mn was in soil P (260 ± 11 mg·kg$^{-1}$; $p < 0.001$). Acceptable limits for Al and Mn in agricultural soils have not been established.

The concentrations of Cd, Cu, Pb, and Zn were highest in soil F, and approached or exceeded the Canadian Soil Quality Guidelines (Table 1). The only soil that met the guideline for Zn was soil P (Table 1). Because the concentrations of Cd, Cu, Pb, and (or) Zn exceeded the CCME (2006) guidelines in at least one of the soil types, these four metals were chosen for screening level risk assessment.

### Metal content of vegetable samples

Multiple analyses of variance (MANOVA), including all 12 elements (Al, Ba, Cd, Co, Cr, Cu, Fe, Mn, Mo, Pb, Se, and Zn) measured in the soil, showed significant site effects on the mean vectors of the edible tissue metal data (Wilks' $\lambda = 0.63$; $F_{[3,48]} = 9.06$; $p < 0.0001$). Vegetables grown in soils A and F had higher concentrations of metals than did those grown in the other two soils. Multiple analyses of variance also detected significant species effects on the mean vectors of the edible tissue metal data (Wilks' $\lambda = 0.17$; $F_{[12,128]} = 9.75$; $p > 0.0001$). The metal content in edible tissues increased in the order of soybean < wheat < lettuce < radish < carrot.

The results of vegetable analyses for Cd, Pb, Zn, and Cu are shown in Table 2. The soil types are presented in order of increasing metal content (P < L < A < F), a pattern which was consistent across all metals. The concentrations of metals in the vegetables generally increased with increasing metal in the soil. Soybeans germinated in soil F, but the seedlings did not survive past 4 d. A second set of seeds was sown, but these seedlings also died.

Although soil F was the only soil with concentrations of Cd above the CCME (2006) limit (Table 1), concentrations of Cd in carrot, radish, and lettuce grown in both soils A and F were well above the Codex limit (CAC 2007) for human consumption. Wheat and soybean grown in soil L also exceeded the limit for Cd. Although all soils were well below the CCME (2006) limit for Pb in agricultural soil, carrot, and radish grown in each of the field soils were at or above the Codex limit (CAC 2007) for consumption. Concentrations of Pb in wheat and soybean were below the detection limit. The concentrations of Zn in carrot, radish, and lettuce grown in soils A and F were an order of magnitude higher than the concentrations of Zn in wheat and soybean grown in those soils. Concentrations of Zn in radish and soybean grown in soil P were higher than expected, given the low concentration of Zn in this soil. The results for Cu are very interesting. Although the mean concentrations for Cu in soils P, L, and A ranged from 21 to 26 mg·kg$^{-1}$, respectively (Table 1), the concentrations of Cu in carrot, wheat, and soybean grown in these soils ranged over 3 orders of magnitude. Codex limits for Zn and Cu in food have not been established as they are essential micronutrients.

In general, the magnitude of BCF was Pb < Zn < Cu < Cd (Fig. 1). The very high BCF values for carrot, radish, and lettuce grown in soil P are a result of Cd being 0.3 to 1.3 mg·kg$^{-1}$ in the plant tissues and the concentration of Cd in soil P being below the detection limit. To calculate these BCFS, one half the detection limit for Cd (0.001 mg·kg$^{-1}$) was used for the soil concentration. In soil P, a very high proportion of the Cd must have been bioaccessible. Among the field soils, the only BCF values for Cd that were above 1 were for carrot and radish grown in soil A (Fig. 1A). The BCF for Cd did not vary among vegetables in soil L, but carrot concentrated Cd to a greater extent than did both radish and lettuce in each of soils A and F. While all BCFs for Pb were below 0.1, the highest BCF for Pb was for carrot grown in soil A (Fig. 1B). No Pb accumulated in seeds of wheat or soybean grown in any soil type. For Zn, all of the BCFs were below 0.12; the highest value was for carrot

**Table 1.** Selected physicochemical properties for each of the four soils.

| Soil | pH* | OM (%) | Sand (%) | Silt (%) | Clay (%) | Conductivity ($\mu S \cdot cm^{-1}$) | Cd ($mg \cdot kg^{-1}$) | Cu ($mg \cdot kg^{-1}$) | Pb ($mg \cdot kg^{-1}$) | Zn ($mg \cdot kg^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| P | 6.5±0.01a | 28.8±0.6a | 70±5a | 25±2c | 5±1b | 396.7±2.1a | ND | 21.4±5.0b | 4.2±1.1c | 135±11c |
| L | 6.8±0.1a | 6.8±0.1c | 24±3b | 48±2b | 28±5a | 250.0±3.2b | 0.3±0.1b | 23.4±1.2b | 10.5±0.3b | 227±9c |
| A | 5.1±0.1c | 7.0±0.01c | 22±2b | 69±4a | 9±3b | 83.2±2.2d | 0.8±0.1b | 25.8±0.7b | 14.9±2.0b | 1211±61b |
| F | 5.8±0.1b | 11.6±0.1b | 80±3a | 16±4c | 4±2b | 198.3±4.6c | 6.3±0.3a | 45.6±1.1a | 40.1±0.7a | 6807±131a |
| CCME limit† | | | | | | | 1.4 | 63 | 70 | 200 |

**Note:** ND, not detected (detection limits in $\mu g \cdot kg^{-1}$: Cd, 1.5; Cu, 1.5; Pb, 15; Zn, 5)

*Within each column, means sharing the same letter are not significantly different (one-way ANOVA followed by Tukey's test, ($p < 0.05$). Values are mean ± SE, $n = 3$.

†The Canadian Council of Ministers of the Environment limits for agricultural soil are presented for comparison (CCME 2006).

**Table 2.** Concentrations of metals in edible tissues of five plant species grown in each of the four soils.

| | Concentration of metal [$mg \cdot kg(\text{dry mass})^{-1}$] | | | | |
|---|---|---|---|---|---|
| | Soil type | | | | |
| | P | L | A | F | Codex limit* |
| **Cd** | | | | | |
| Carrot | 0.7±0.3b | 0.8±0.2b | 38.6±19.9a | 42.5±11.1a | 1 |
| Radish | 1.3±0.8b | 1.9±0.7b | 11.0±3.8b | 13.3±1.5a | 1 |
| Lettuce | 0.3±0.01b | 1.6±0.3b | 3.7±3.4b | 8.2±1.4a | 2 |
| Wheat | ND | 0.5±0.02a | 0.06±0.06c | 0.2 ±0.004b | 0.22 |
| Soybean | ND | 0.3±0.01a | ND | - - | 0.11 |
| **Pb** | | | | | |
| Carrot | 0.3±0.2b | 1.0±0.5b | 12.2±3.1a | 6.5±2.2ab | 1 |
| Radish | 0.9±0.9a | 2.5±1.3a | 3.8±1.3a | 3.9±2.2a | 1 |
| Lettuce | 1.9±0.2a | 0.7±0.7a | 2.6±1.3a | 2.8±1.6a | 3 |
| Wheat | ND | ND | ND | ND | 0.22 |
| Soybean | ND | ND | ND | - - | 0.22 |
| **Zn** | | | | | |
| Carrot | 41.0±10.2a | 25.9±4.3a | 1401.0±199a | 2737±734a | |
| Radish | 52.0±7.7c | 23.2±6.8c | 955.0±5b | 1890±560a | |
| Lettuce | 24.9±8.2b | 30.1±7.2b | 372.0±20b | 1350±76a | |
| Wheat | 16.8±0.6b | 22.6±1.8b | 32.6±14.4b | 168±11.3a | |
| Soybean | 56.5±0.4a | 39.8±3.1b | 42.3±0.8b | - - | |
| **Cu** | | | | | |
| Carrot | 6.5±1.3c | 8.4±4.3c | 35.6±3.7a | 21.2±2.6b | |
| Radish | 5.2±1.6b | 9.1±3.5b | 19.7±7.8b | 27.9±2.7a | |
| Lettuce | 3.0±1.2a | 4.6±0.4a | 4.6±0.6a | 7.1±2.8a | |
| Wheat | 0.7±0.7b | 60.4±3.0a | 122.0±54a | 83.2±1.0a | |
| Soybean | 4.4±0.8c | 41.9±2.2a | 32.9±0.8b | - - | |

**Note:** Within each row, means sharing the same letter are not significantly different (one-way ANOVA followed by Tukey's test, $p < 0.05$) Values are mean ± SE, $n = 3$. ND, not detected (detection limits in $\mu g \cdot kg^{-1}$: Cd, 1.5; Cu, 1.5; Pb, 15; Zn, 5); - - = soybean did not survive in soil F.

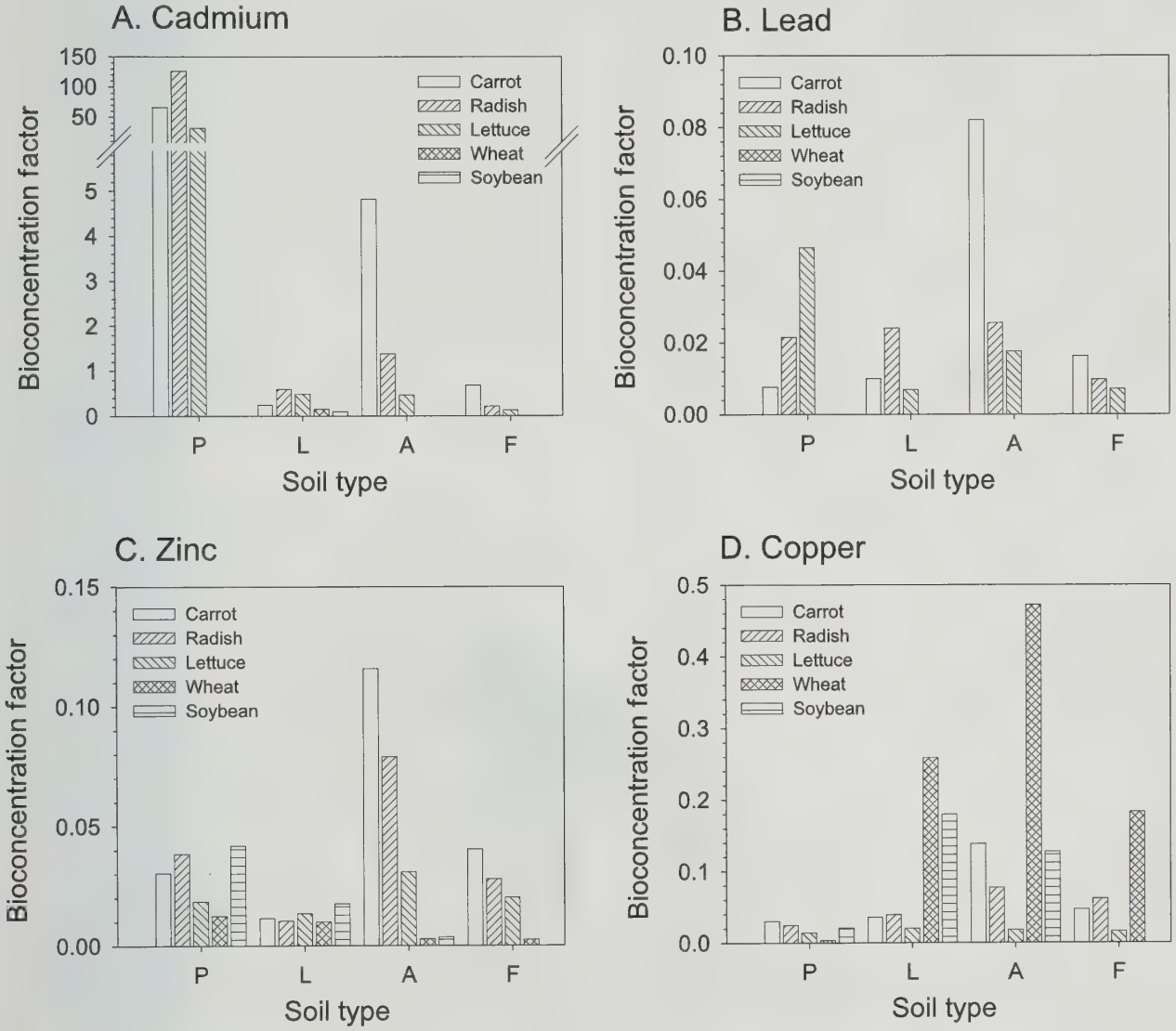
*The Codex Alimentarius Commission standards (CAC 2007) for metal content in the vegetables are presented for comparison. These Codex limits have been converted from $mg \cdot kg(\text{fresh mass})^{-1}$ to $mg \cdot kg(\text{dry mass})^{-1}$ using a wet:dry ratio of 10:1 for root and leaf tissues, and a wet:dry ratio of 10:9 for seeds; these values correspond to average in-lab measurements before and after drying. Codex limits have not yet been established for Zn and Cu.

grown in soil A (Fig. 1C). The BCFs for Cu were below 0.5 for all vegetables in each of the field soils (Fig. 1D). For Cu, the highest BCFs were recorded for wheat grown in the three field soils.

### Screening level risk evaluation to human health

The results of the human health screening level risk assessment for females are shown in Fig. 2. The data for males were similar, but owing to their higher average body mass the HQ values for males were 95% those of the females (data not shown). In terms of Cd (Fig. 2A), consumption of neither wheat nor soybean would pose a risk (all HQ below 0.1); however, carrot, radish, and lettuce grown in soils A and F, and radish grown in soil L had HQs well above 1 (range: 1.5–37). No vegetable presented a risk with respect to consuming an unacceptable amount of Pb; the highest HQ was only 0.3 (Fig. 2B). Carrot and radish grown in soil A, and carrot radish and lettuce grown in soil F had HQ values for Zn ranging from 2 to 8 (Fig. 2C). For Cu, only wheat grown in soil A had an HQ above 1 (Fig. 2D).

**Fig. 1.** Bioconcentration factors (BCFs) of vegetables grown in the four soils. BCF = $M_c / M_s$ (concentration of metal in the edible tissue / concentration of metal in the soil). Soil type: P, Promix; L, London agricultural soil; A, Quebec agricultural soil; F, Quebec forest soil.




## Discussion

### Soil characterization

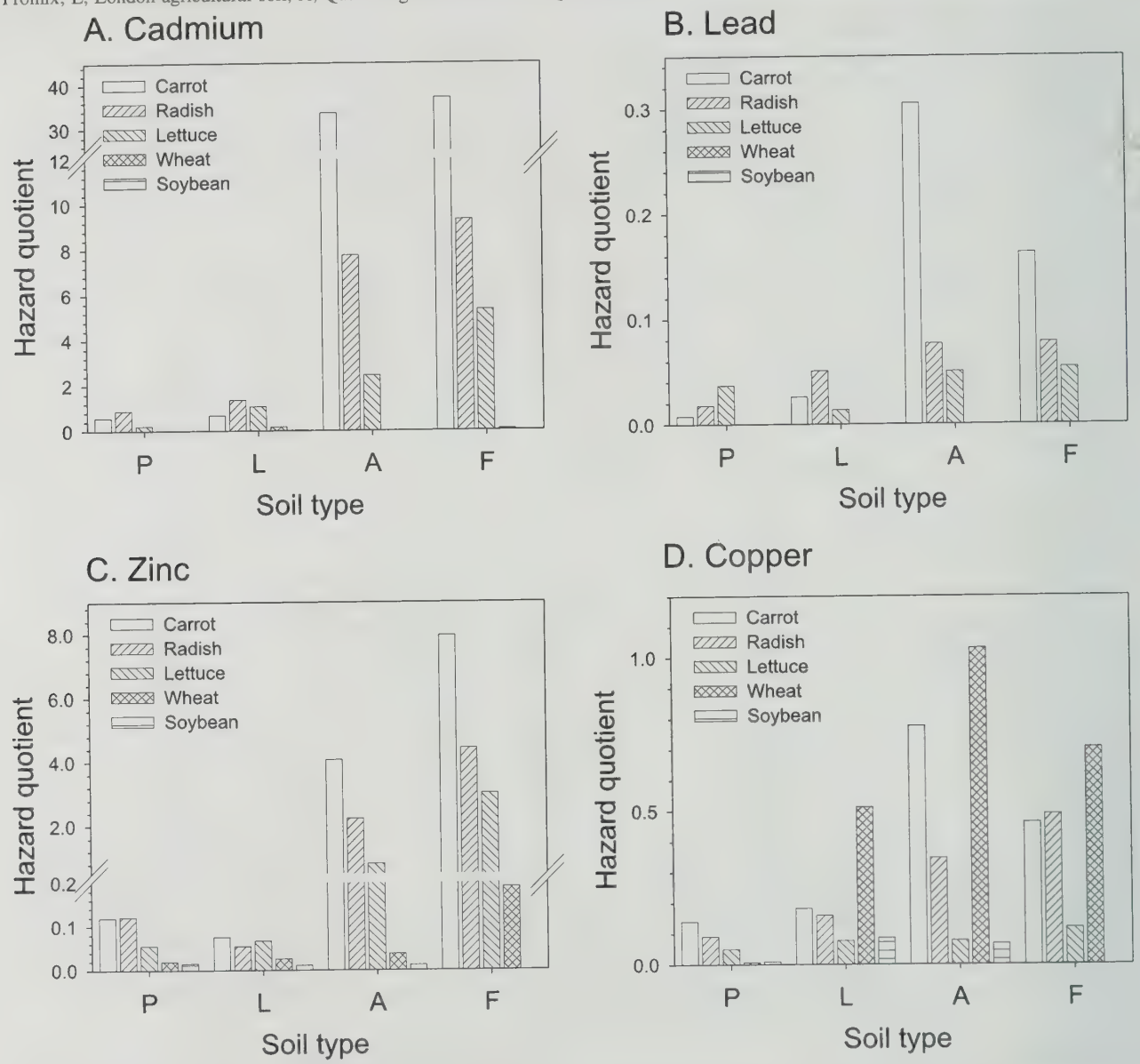
The pH and percent organic matter in the field soils are typical of agricultural soils in eastern Canada (Gregorich et al. 1995). Given the lower pH of soils A and F, one might expect metals in these soils to be more bioaccessibile (Evans et al. 1995; Peijnenburg and Jager 2003; Sinha et al. 2006); however, these two soil also have a lower conductivity, which could result in reduced metal bioavailability (Shuman 1999). Soil L had a higher proportion of clay and a higher pH relative to soils A and F, thus plants grown in soil L might be expected to have lower metal content. To examine metal bioavailability in more detail, two variables were considered: the influence of soil type and the influence of plant species.

### The influence of soil type

Generally, metal content in the vegetables increased with increasing metal in the soil and with decreasing soil pH, although this pattern was not true for some plant–soil combinations. No consistent relationship between metal type, BCF and soil type was observed, which is in contrast with Alam et al. (2003) who found BCF values for As decreased with increasing soil metal.

If pH were the dominant variable in terms of metal bioavailability, then the BCFs should be highest in soil A and second highest in soil F. Although this pattern of BCF was not observed for all metals within any one plant species, nor for any one metal across plant species, the soil with the lowest pH (A) did yield the highest BCF for Cd, Pb, and Zn in

**Fig. 2.** Hazard quotients (HQ) of vegetables grown in the four soils. HQ is an estimate of the risk associated with consumption of each vegetable (a ratio of the amount of metal consumed relative to the maximum amount of metal deemed to be nonhazardous). Soil type: P, Promix; L, London agricultural soil; A, Quebec agricultural soil; F, Quebec forest soil.




carrot, for Zn in radish and for Cu in wheat. Furthermore, metals such as Cd and Cu tend to precipitate out of the soil solution at pH values ranging from 6–8 (Kamnev and van der Lelie 2000; Ismail et al. 2005), suggesting that soils L and P should have a smaller proportion of these metals available for uptake. However, in radish the highest BCF for Pb was seen for plants grown in soil P and wheat grown in soil L had the second highest BCF for Cu.

Soil texture, conductivity, and OM could also contribute to a difference in bioavailability of metal in the soils. Of the three field soils, soil F had the lowest clay content, the highest OM content, and a mid-range conductivity, all of which should result in decreased metal bioavailability (Ali et al. 2004). However, as was seen for pH, no clear relationship between these variables and metal uptake within a plant species, or across plant species, was seen.

Of course, it is reasonable to believe that some combination of physicochemical characteristics determines metal bioaccessibility within a soil, and different conditions may differentially affect different metals. It could be that the range of available data was too narrow or an insufficient number of variables were measured in this study to clearly identify the relationship between soil type and metal accumulation in the plants. Nonetheless, if the physicochemical

characteristics of a soil lead to increased bioaccessibilty of at least one metal, one might expect many plants grown in that soil to accumulate that metal. This was not seen. For example, in soil A, only carrot had high BCFs for Cd and Pb and wheat had the highest BCF for Cu. Clearly, species-specific variables are as important, or more important, than soil characteristics in terms of regulating metal bioavailability.

Notwithstanding the above, it is important to note that BCF values obtained from potted plant experiments may overestimate BCFs relative to plants grown in the field because metal bioaccessibility is often higher in soils that have been dried and stored (reviewed in Bartlett and James 1980). In addition, plants in our study were grown at 25 °C, which may have resulted in increased metal bioaccessibility relative to plants grown in cooler field soil (Antoniadis and Alloway 2001). On the other hand, some of the larger wheat and soybean in our experiment became pot-bound. Had they been permitted to explore a larger volume of soil, they may have accumulated higher concentrations of metals.

### The influence of species

The relationship between soil characteristics and vegetable species is also illustrated by the BCFs. Overall, carrot showed the highest potential for Cd, Pb, and Zn uptake, whereas wheat accumulated Cu to greater extents than the other vegetables did. The high BCFs for carrot are surprising, since Intawongse and Dean (2006) found that concentrations of Cd, Cu, Mn, and Zn were higher in carrot leaves than in carrot roots. Others have reported that roots generally contain elevated concentrations of metals than do shoots, owing to retention of metals in the root (reviewed in Clemens et al. 2002). Radish is also a root crop and, while concentrations of Cd and Zn were higher in radish than in the nonroot crops, the concentrations of Zn and Cu were sometimes as high, or higher, in wheat and soybean than they were in radish. In our study, lettuce accumulated relatively low concentrations of each metal. This is in contrast to many other reports in which leafy vegetables (especially lettuce and spinach) were shown to accumulate metals to a greater extent than some other vegetable types (Qadir et al. 2000; Harrison 2001; Alexander et al. 2006).

Within each soil, accumulation of metals varied among plant species. The differences in the BCFs for each metal among the five vegetables suggest that plant species differ in uptake and translocation abilities. In addition, because no one vegetable consistently took up any one metal from each soil, net bioavailability must be the result of plant–soil interactions. Our results are in agreement with Jung and Thornton (1996), Alexander et al. (2006), Intawongse and Dean (2006), and Sipter et al. (2008) who found that metal concentrations in plants differed with species, and that the pattern among species varied among the different elements.

### Screening level risk assessment

As expected, all vegetables grown in soils P and L had very low HQs; these soils were below the CCME (2006) limits for Cd, Pb, and Cu, and soil L was only slightly above the limit for Zn. In addition, each soil was well below the CCME (2006) limit for Pb and no vegetable accumulated Pb to a potentially unsafe level. It is also not surprising that carrot, radish, and lettuce grown in soil F had high HQs for Cd and Zn; that soil had four times the CCME (2006) limit for Cd and 30 times the limit for Zn. Similarly, soil A had six times the limit for Zn, and each of carrot, radish, and lettuce had HQs >1 for Zn. Muchuweti et al. (2006) reported unsafe concentrations of Cd, Cu, Pb, and Zn in six dietary crops that were grown in soils that exceeded the United Kingdom regulations for these metals. In general, soils that exceed quality guidelines offer the potential for a contaminated crop.

However, elevated concentrations of metal in a soil do not necessarily result in high HQs; for example, the HQs for Zn were low for vegetables grown soil L, which was 10% over the CCME (2006) limit for Zn. Sipter et al. (2008) also reported nontoxic concentrations of metals in vegetables that were grown in contaminated soil. At the same time, growth in an apparently "uncontaminated" soil can result in a potentially unsafe crop. For example, Cd in soil A was well below the CCME (2006) limit, yet the HQs for Cd in these vegetables ranged from 2 to >30.

Our HQ calculations were based on the assumption that consumers ate only one type of vegetable in their diet and the vegetable was eaten raw. This was intended to represent the worst case scenario of eating produce from these soils. In reality, most people eat a mixed vegetable diet and some methods of vegetable preparation (e.g., boiling) may leach metals prior to consumption. Nonetheless, our results indicate that soil-use guidelines should not be based solely on the concentrations of metals in soil but also other soil properties and on crop species. For example, guidelines might include a list of "low accumulating" crops that might be suitable for growth on mildly contaminated soils (Alexander et al. 2006).

## Conclusions

No generalized pattern of metal uptake was observed either between soil types or between plant species. That is to say that no single soil type or plant species was observed to hold the highest influence on metal uptake; rather, metal uptake was observed to vary depending on soil, plant species, and the metal in question. This indicates the need for a more thorough investigation of the influences of plant–soil interactions on metal uptake, and integration of the findings into risk assessment models.

## Acknowledgements

The authors gratefully acknowledge the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant Program and the Canadian NSERC Metals in the Human Environment (MITHE) Strategic Network (a full list of sponsors is available at www.mithe-sn.org). We also thank Dr. François Courchesne and Mr. Ron Smith for supplying field soils, and Dr. Charles Wu for ICP-AES analyses.

## References

Alam, M.G.M., Snow, E.T., and Tanaka, A. 2003. Arsenic and heavy metal contamination of vegetables grown in Samta village, Bangladesh. Sci. Total Environ. **308**: 83–96. doi:10.1016/S0048-9697(02)00651-4. PMID:12738203.

Alexander, P.D., Alloway, B.J., and Dourado, A.M. 2006. Genotypic variations in the accumulation of Cd, Cu, Pb and Zn exhibited by six commonly grown vegetables. Environ. Pollut. **144**: 736–745. doi:10.1016/j.envpol.2006.03.001. PMID:16647172.

Ali, N.A., Ater, M., Sunahara, G.I., and Robidoux, P.Y. 2004. Phytotoxicity and bioaccumulation of copper and chromium using barley (*Hordeum vulgare* L.) in spiked artificial and natural forest soils. Ecotoxicol. Environ. Saf. **57**: 363–374. doi:10.1016/S0147-6513(03)00074-5. PMID:15041259.

Antoniadis, V., and Alloway, B.J. 2001. Availability of Cd, Ni and Zn to ryegrass in sewage sludge-treated soils at different temperatures. Water Air Soil Pollut. **132**: 201–214. doi:10.1023/A:1013202104550.

Antunes, P.M.C., Berkelaar, E.J., Boyle, D., Hale, B.A., Hendershot, W., and Voight, A. 2006. The biotic ligand model for plants and metals: technical challenges for field application. Environ. Toxicol. Chem. **20**: 2561–2571.

Bartlett, R. and James, B. 1980. Studying dried, stored soils samples — some pitfalls. Soil Sci. Soc. Am. **44**: 721–724.

Bradl, H.B. 2004. Adsorption of heavy metal ions on soils and soils constituents. J. Colloid Interface Sci. **277**: 1–18. doi:10.1016/j.jcis.2004.04.005. PMID:15276031.

Brazauskiene, D., Paulauskas, V., and Sabiene, N. 2008. Speciation of Zn, Cu, and Pb in the soil depending on soil texture and fertilization with sewage sludge compost. J. Soils Sediments, **8**: 184–192. doi:10.1007/s11368-008-0004-6.

CAC. 2007. Codex standard #193–1995: general standard for contaminants and toxins in foods. [Online]. Available from www.codexalimentarius.net/web/standard_list.do [accessed 23 October 2008].

CCME. 2006. Canadian environmental quality guidelines. [Online]. Available from www.ccme.ca/publications/ceqg_rcqe.html [accessed 21 October 2008].

Clemens, S., Palmgren, M.G., and Kramer, U. 2002. A long way ahead: understanding and engineering plant metal accumulation. Trends Plant Sci. **7**: 309–315. doi:10.1016/S1360-1385(02)02295-1. PMID:12119168.

Diaz-Zorita, M., Perfect, E., and Grove, J.H. 2002. Disruptive methods for assessing soil structure. Soil Tillage Res. **64**: 3–22. doi:10.1016/S0167-1987(01)00254-9.

Evans, L.J., Spiers, G.A., and Zhao, G. 1995. Chemical aspects of heavy metal solubility with reference to sewage sludge amended soils. Int. J. Environ. Anal. Chem. **59**: 291–302. doi:10.1080/03067319508041335.

Ge, Y., and Hendershot, W. 2005. Modeling sorption of Cd, Hg and Pb in soils by the NICA-Donnan Model. Soil Sediment Contam. Int. J. **14**: 53–69.

Gregorich, E.G., Angers, D.A., Campbell, C.A., Carter, M.R., Drury, C.F., Ellert, B.H., Groenevelt, P.H., Holmstrom, D.A., Monreal, C.M., Rees, H.W., Voroney, R.P., and Vyn, T.J. 1995. Changes in soil organic matter. *In* The health of our soils: toward sustainable agriculture in Canada. *Edited by* D.F. Acton and L.J. Gregorich. Agriculture and Agri-Food Canada Publication 1906/E.

Harrison, P. 2001. Health effects of environmental chemicals. *In* Pollution: causes, effects and control. *Edited by* R.M. Harrison. Royal Society of Chemistry, Cambridge pp. 500–523.

Health Canada. 2008. Canada food guide. [Online]. Available from www.healthcanada.gc.ca/foodguide [accessed 30 September 2008].

Heiri, O., Lotter, A.F., and Lemeke, G. 2001. Loss in ignition as a method for estimating organic and carbonate content in sediments: reproducibility and comparability of results. J. Paleolimnol. **25**: 101–110. doi:10.1023/A:1008119611481.

Hoagland, D.R., and Arnon, D.I. 1950. The water culture method for growing plants without soil, Calif. Agric. Exp. Stn. Circ. 1–32.

Ingwersen, J., and Streck, T. 2005. A regional-scale study on the crop uptake of cadmium from sandy soils: measurement and modeling. J. Environ. Qual. **34**: 1026–1035. doi:10.2134/jeq2003.0238. PMID:15888888.

Intawongse, M., and Dean, J.R. 2006. Uptake of heavy metals by vegetable plants grown on contaminated soil and their bioavailability in the human gastrointestinal tract. Food Addit. Contam. **23**: 36–48. doi:10.1080/02652030500387554. PMID:16393813.

Ismail, B.S., Farihah, K., and Khairiah, J. 2005. Bioaccumulation of heavy metals in vegetables from selected agricultural areas. Bull. Environ. Contam. Toxicol. **74**: 320–327. doi:10.1007/s00128-004-0587-6. PMID:15841973.

Jung, M.C., and Thornton, I. 1996. Heavy metal concentration of soils and plants in the vicinity of lead–zinc mine, Korea. Appl. Geochem. **11**: 53–59. doi:10.1016/0883-2927(95)00075-5.

Kamnev, A.A., and van der Lelie, D. 2000. Chemical and biological parameters as tools to evaluate and improve heavy metal phytoremediation. Biosci. Rep. **20**: 239–258. doi:10.1023/A:1026436806319. PMID:11092247.

Kasuya, M. 2000. Recent epidemiological studies on Itai-itai disease as a chronic cadmium poisoning in Japan. Water Sci. Technol. **42**: 147–154.

McBride, M.B. 1989. Reactions controlling metal solubility in soils. Adv. Soil Sci. **10**: 1–56.

MITHE-SN. 2007. Quality assurance/quality control. [Online]. Available from www.mithern.org/data_management/qa_qc.cfm [accessed 30 September 2008].

Muchuweti, M., Birkett, J.W., Chinyanga, E., Zvauya, R., Scrimshaw, M.D., and Lester, J.N. 2006. Heavy metal content of vegetables irrigated with mixtures of wastewater and sewage sludge in Zimbabwe: implications for human health. Agric. Ecosyst. Environ. **112**: 41–48. doi:10.1016/j.agee.2005.04.028.

Page, V., Weisskopf, L., and Feller, U. 2006. Heavy metals in white lupin: uptake, root-to-shoot transfer and redistribution within the plant. New Phytol. **171**: 329–341. doi:10.1111/j.1469-8137.2006.01756.x. PMID:16866940.

Peijnenburg, W.J.G.M., and Jager, T. 2003. Monitoring approaches to assess bioaccessibility and bioavailability of metals: matrix issues. Ecotoxicol. Environ. Saf. **56**: 63–77. doi:10.1016/S0147-6513(03)00051-4. PMID:12915141.

Pierzynski, G.M., Sims, J.T., and Vance, G.F. 2000. Soils and environmental quality, 2nd ed. CRC Press, Boca Raton, Fla.

Qadir, M., Ghafoor, A., and Murtaza, G. 2000. Cadmium concentration in vegetables grown on urban soils irrigated with untreated municipal sewage. Environ. Dev. Sustain. **2**: 11–19. doi:10.1023/A:1010061711331.

Rieuwerts, J., Thornton, I., Farago, M., and Ashmore, M. 1998. Quantifying the influence of soil properties on the solubility of metals by predictive modeling of secondary data. Chem. Spec. Bioavail. **10**: 83–94. doi:10.3184/095422998782775817.

Ross, S.M. 1994. Sources and forms of potentially toxic metals in soil–plant systems. *In* Toxic metals in soil–plant systems. *Edited by* S.M. Ross. John Wiley and Sons, London, UK.

Sauvé, S., Dumestre, A., McBride, M., Gillet, J.W., Berthelin, J., and Hendershot, W. 1999. Nitrification potential in field-collected soils contaminated with Pb or Cu. Appl. Soil Ecol. **12**: 29–39. doi:10.1016/S0929-1393(98)00166-8.

Sauvé, S., Norvell, W.A., McBride, M., and Hendershot, W. 2000. Speciation and complexation of cadmium in extracted soil solutions. Environ. Sci. Technol. **34**: 291–296.

Shuman, L. 1999. Effect of organic waste amendments on zinc ad-

sorption by two soils. Soil Sci. **164**: 197–205. doi:10.1097/00010694-199903000-00006.

Shute, T., and Macfie, S.M. 2006. Cadmium and zinc accumulation in soybean: a threat to food safety? Sci. Total Environ. **371**: 63–73. doi:10.1016/j.scitotenv.2006.07.034. PMID:16949649.

Sinha, S., Gupta, A.K., Bhatt, K., Pandey, K., Rai, U.N., and Singh, K.P. 2006. Distribution of metals in the edible plants grown at Jajmau, Kanpur (India) receiving treated tannery wastewater: relation with physico-chemical properties of the soil. Environ. Monit. Assess. **115**: 1–22. doi:10.1007/s10661-006-5036-z. PMID:16502026.

Sipter, E., Rozsa, E., Gruiz, K., Tatrai, E., and Moravai, V. 2008. Site-specific risk assessment in contaminated vegetable gardens. Chemosphere, **71**: 1301–1307. doi:10.1016/j.chemosphere.2007.11.039. PMID:18191173.

Tack, F., and Verloo, M. 1995. Chemical speciation and fractionation in soil and sediment heavy-metal analysis: a review. Int. J. Environ. Anal. Chem. **59**: 225–238. doi:10.1080/03067319508041330.

Taylor, G.J., Blamey, F.P.C., and Edwards, D.G. 1998. Antagonistic and synergistic interactions between aluminum and manganese on growth of *Vigna unguiculata* at low ionic strength. Physiol. Plant. **104**: 183–194. doi:10.1034/j.1399-3054.1998.1040206.x.

US EPA. 2005. Test methods for evaluating solid waste, physical/chemical methods, series 3000. [Online]. Available from www.epa.gov/epaoswer/hazwaste/test/main.htm [accessed 27 October 2008].

US EPA. 2006. Integrated risk information system (IRIS). [Online]. Available from www.epa.gov/iris/ [accessed 20 October 2008].

USDA. 2008. Soil texture calculator. [Online]. Available from soils.usda.gov/technical/aids/investigations/texture/ [accessed 28 October 2008].

Van Assche, F., and Clijsters, H. 1990. A biological test system for the evaluation of the phytotoxicity of metal-contaminated soils. Environ. Pollut. **66**: 157–172. doi:10.1016/0269-7491(90)90118-V. PMID:15092244.

# Hypobaria, hypoxia, and light affect gas exchange and the $CO_2$ compensation and saturation points of lettuce (*Lactuca sativa*)[1]

**Chuanjiu He, Fred T. Davies Jr., and Ronald E. Lacey**

**Abstract:** There are important engineering and crop production advantages in growing plants under hypobaric (reduced atmospheric pressure) conditions for extraterrestrial base or spaceflight environments. The objectives of this research were to determine the influence of hypobaria and reduced partial pressure of oxygen ($pO_2$) (hypoxia) under low and high light irradiance on carbon dioxide ($CO_2$) assimilation ($C_A$), dark-period respiration (DPR), and the $CO_2$ compensation and $CO_2$ saturation points of lettuce (*Lactuca sativa* L. 'Buttercrunch'). Plants were grown under variable total gas pressures [25 and 101 kPa (ambient)] at 6, 12, or 21 kPa $pO_2$ (approximately the partial pressure in air at normal pressure). Light irradiance at canopy level of the low-pressure plant growth system (LPPG) was at 240 (low) or 600 (high) $\mu mol \cdot m^{-2} \cdot s^{-1}$. While hypobaria (25 kPa) had no effect on $C_A$ or the $CO_2$ compensation point, it reduced the DPR and the $CO_2$ saturation point, and increased the $C_A$ / DPR ratio. Hypoxia (6 kPa $pO_2$) and low light reduced $C_A$, DPR, and the $C_A$ / DPR ratio. Hypoxia decreased the $CO_2$ compensation point regardless of total pressure. Hypoxia also decreased the the $CO_2$ saturation point of ambient-pressure plants, but had no effect on hypobaric plants. While low light reduced the $CO_2$ saturation point, it increased the $CO_2$ compensation point, compared with high-light plants. The results show that hypobaric conditions of 25 kPa do not adversely affect gas exchange compared with ambient-pressure plants, and may be advantageous during hypoxic stress.

*Key words:* $CO_2$ compensation point, $CO_2$ saturation point, hypobaria, hypoxia, low-pressure plant growth system.

**Résumé :** Il y a d'importants avantages d'ingénierie et de production de culture à faire pousser les plantes en conditions hypobares (pression atmosphérique réduite) pour les environnements des vols dans l'espace et des bases extraterrestres. Le but de cette recherche consistait à déterminer l'influence de l'hypobarie et d'une réduction partielle de la pression en oxygène ($pO_2$) [hypoxie], sous des irradiances faibles ou fortes, sur l'assimilation ($C_A$) du bioxyde de carbone ($CO_2$), la respiration en phase obscure (DPR), ainsi que la compensation en $CO_2$ et les points de saturation en $CO_2$ de la laitue (*Lactuca sativa* L. 'Buttercrunch'). Les auteurs ont cultivé les plantes sous des pressions variables en gaz totaux [25 et 101 kPa (ambiant)] et 6, 12 ou 21 kPa $pO_2$ (environ la pression partielle dans l'air à pression normale). L'irradiance lumineuse à la hauteur de la canopée dans le système de culture à faible pression (LPPG), était de 240 (faible) ou 600 (forte) $\mu mol \cdot m^{-2} \cdot s^{-1}$. Alors que l'hypobarie (25 kPa) demeure sans effet sur le $C_A$ ou le point de compensation, elle réduit le DPR du point de saturation en $CO_2$, et augmente le rapport $C_A$ / DPR. L'hypoxie (6 kPa $pO_2$) et la faible illumination réduisent le $C_A$, le DPR et le rapport $C_A$ / DPR. L'hypoxie diminue le point de compensation en $CO_2$ indépendamment de la pression totale. L'hypoxie réduit également le point de saturation en $CO_2$ chez les plantes Á pression ambiante, mais demeure sans effet sur les plantes en conditions hypobare. Bien que la faible intensité lumineuse réduise le point de saturation du $CO_2$, elle augmente le point de compensation en $CO_2$, comparativement aux plantes à haute intensité lumineuse. Les résultats montrent que les conditions hypobares de 25 kPa n'affectent pas de façon adverse l'échange des gaz comparativement aux plantes sous pression ambiante, et pourraient constituer un avantage au cours de stress hypoxiques.

*Mots-clés :* point de compensation du $CO_2$, point de saturation en $CO_2$, hypobarie, hypoxie, système de culture de plantes à faible pression.

[Traduit par la Rédaction]

Received 31 October 2008. Published on the NRC Research Press Web site at botany.nrc.ca on 15 July 2009.

**C. He and F.T. Davies, Jr.**[2] Department of Horticultural Sciences and Interdisciplinary Program of Molecular and Environmental Plant Sciences (MEPS), College Station, TX 77843-2133, USA.
**R.E. Lacey.** Department of Biological and Agricultural Engineering, Texas A&M University, College Station, TX 77843-2117, USA.

[1]This paper is one of a selection published in a Special Issue comprising papers presented at the 50th Annual Meeting of the Canadian Society of Plant Physiologists (CSPP) held at the University of Ottawa, Ontario, in June 2008.
[2]Corresponding author (e-mail: f-davies@tamu.edu).

 doi:10.1139/B09-031 

## Introduction

The exploration of space requires the development of advanced life support systems (ALS) that have the capacity to recycle resources and produce food (Bugbee and Salisbury 1989*a*, 1989*b*; Wheeler et al. 2001). The biological component will include the use of higher plants for supplemental air and water purification, as well as providing food and psychological benefits (NASA 1998). Plants grown in space environments will be subject not only to reduced gravity, but also to the conditions designed mainly to fulfill requirements for the human environment (Wheeler et al. 2001). Plants can tolerate wide variation in the concentration of the essential gases $O_2$ and $CO_2$, relative humidity, and possibly also, total gas pressure (Wheeler et al. 1994; Corey et al. 2002; Spanarkel and Drew 2002; He et al. 2003, 2006). An important environmental variable that has received limited research effort in relation to an ALS is reduced total atmospheric gas pressure at elevated $CO_2$ partial pressure ($pCO_2$).

There are several important advantages, from an engineering and cost-reduction viewpoint, associated with growing plants at hypobaric conditions for biomass production in controlled lunar or martian extraterrestrial base environments. The reduced pressure differential between the plant-growth facility and the external environment would reduce structural requirements, permit lighter materials to be used in its construction, and improve safety issues by reducing external and internal pressure differentials. This would further reduce the payload volume and mass required for deployment. A hypobaric condition would reduce gas leakage from the controlled environment space to the external environment. For a lunar mission, the ambient pressure is near 0 kPa, whereas the martian ambient pressure varies from 0.2 to 0.9 kPa. Pressure differences between the hypobaric lunar and martian environment, and maintaining a higher, earth-ambient total pressure, would result in greater leakage. Furthermore, hypobaric conditions in the plant growth facility would require less buffer gas, typically $N_2$, to be transported or obtained in situ to supplement the physiologically active gases ($CO_2$ and $O_2$).

Earlier studies have demonstrated that seed germination and seedling growth are possible at hypobaric conditions (Gale 1973; Musgrave et al.1988; Schwartzkopf and Mancinelli 1991; Andre and Massimino 1992; Corey et al. 2002; Spanarkel and Drew 2002; He et al. 2003). It is also a common observation that plants can be grown at high altitude, where pressures are well below 70 kPa (Gale 1972; Davies et al. 2005), although the invariable association between increasing altitude and decreasing temperature confounds the issue of the effect of pressure alone. The question here is whether the rates of vegetative growth and morphogenesis compare closely with those at ambient pressure. A major potential limitation to plant growth under hypobaria is if the partial pressure of $O_2$ ($pO_2$) is reduced, oxidative phosphorylation can become limited (Drew 1997). Seedlings germinated and grew during a week-long study at 6 kPa total gas pressure, provided the atmosphere was composed predominately of oxygen ($pO_2$ = 5 kPa; ~83% $O_2$); but at lower total pressures and therefore less $O_2$, seeds failed to germinate (Schwartzkopf and Mancinelli 1991). Low total pressure (21–24 kPa) did not inhibit seed germination and initial growth as long as $pO_2$ was 5 kPa or more (Musgrave et al. 1988). Hypobaric environments are typically associated with hypoxia (low $O_2$), particularly when total gas pressure is reduced below 50 kPa. Hence there is a need to supply sufficient partial pressures of $O_2$ to avoid hypoxia under hypobaric conditions.

Gas-exchange parameters in the leaf are indicators of biochemical processes in mesophyll cells (von Caemmerer and Faquhar 1981). $CO_2$ response curves have been used for determining carboxylation efficiency and factors limiting photosynthesis. From these curves, both $CO_2$ saturation [sat($CO_2$)] and $CO_2$ compensation [$\Gamma(CO_2)$] can be determined, which are important parameters in modeling $CO_2$ assimilation ($C_A$) and dark-period respiration (DPR) in an advanced, closed, life support system. The $\Gamma(CO_2)$ represents the $CO_2$ concentration at which the rate of photosynthetic $CO_2$ uptake is equal to the rate of $CO_2$ released from photorespiration and dark-period respiration (Espie and Colman 1987). With $C_3$ carbon fixation plants such as lettuce, photosynthetic carbon metabolism is affected by the competition between $CO_2$ and $O_2$ for ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). While RuBisCO has a higher affinity for binding $CO_2$, it also has an oxygenase function and can combine $O_2$ to RuBP; hence, if enough $O_2$ is present it can act as a competitive inhibitor via photorespiration. Thus the ratio of $CO_2$ and $O_2$ concentrations in the chloroplast and immediate gaseous environment and the subsequent $O_2$ and $CO_2$ compensation points affects RuBisCO and the fixing of $CO_2$ (Smith et al. 1976; Tolbert et al. 1995). Plants with low rates of respiration and photorespiration, and a high rate of internal $CO_2$ fixation, have low $\Gamma(CO_2)$, such as $C_4$ plants, whereas less efficient terrestrial $C_3$ plants have higher $\Gamma(CO_2)$ (Hough and Wetzel 1978). While factors such as $O_2$ concentration, temperature, and drought stress affect $\Gamma(CO_2)$, little is known about the influence of hypobaria, which has been associated with hypoxia, i.e., plants grown at high altitude. Thus, the objectives of this research were to characterize the effect of hypobaria, hypoxia, and light irradiance on leaf metabolic activities via gas exchange, sat($CO_2$) and $\Gamma(CO_2)$ of lettuce (*Lactuca sativa* L. 'Buttercrunch').

## Materials and methods

### Low pressure plant growth system (LPPG)

The LPPG system is a fully automated system, capable of controlling pressure and gas concentrations in ambient or reduced pressure growth chambers (He et al. 2006, 2007). The LPPG system consisted of six growth chambers designed to operate at pressures as low as 5 kPa (Fig. 1). The six chambers were housed in an environmentally controlled growth room. Total pressures, and partial pressures of oxygen ($pO_2$) and carbon dioxide ($pCO_2$) were controlled and monitored during experiments. The LPPG was a semi-closed system, since $O_2$, $CO_2$, and $N_2$ were added and controlled. Temperature was recorded, although not controlled directly by the LPPG system. Temperature control and lighting were provided by placing the LPPG system in an independent plant growth room. The pressure and control systems of each chamber were independent so that conditions could be set to satisfy any statistical experimental design. The LPPG op-

**Fig. 1.** Low pressure plant growth (LPPG) system with lettuce plants under differential total pressures of 101 and 25 kPa.

erating system and parameters measured are explained in greater detail in He et al. (2007). There was also an ethylene scrubbing system to remove endogenous ethylene generated by lettuce plants. The system included a stainless steel column filled with potassium permanganate to strip ethylene from the air as it is circulated over the cooling coils and returned to a given chamber.

### Plant growth conditions

Lettuce ( 'Buttercrunch') was germinated in 20 cm × 14 cm plastic pots that were filled to within 1 cm of the top with fine-grade calcined clay [Profile Greens, Profile Products LLC, Buffalo Grove, Illinois, USA (particle size < 1 mm, 74% porosity, 0.56 g·mL$^{-1}$ bulk density and 2.5 g·mL$^{-1}$ particle density)]. The inert, calcined clay was prewashed with deionized water and allowed to drain thoroughly before sowing. After the seeds were germinated, plants were supplied with a modified Hoagland's nutrient solution (pH 6.3) containing 4.0 mmol·L$^{-1}$ $Ca(NO_3)_2$, 1.0 mmol·L$^{-1}$ $KH_2PO_4/K_2HPO_4$, 2.0 mmol·L$^{-1}$ $KNO_3$, 1.0 mmol·L$^{-1}$ $MgSO_4$, 50 μmol·L$^{-1}$ Fe as Fe-EDTA, 1 mmol·L$^{-1}$ NaCl, and micronutrients: 50 μmol·L$^{-1}$ B, 10.0 μmol·L$^{-1}$ Mn, 1.0 μmol·L$^{-1}$ Cu, 2.0 μmol·L$^{-1}$ Zn, and 0.3 μmol·L$^{-1}$ Mo.

Ten days after imbibition, three seedlings were transplanted to 20 cm self-watering pots (S-series pots, Apollo Plastics LTD, Mississauga, Ontario, Canada) with a volume of 4 L, filled with pre-washed calcined clay. The reservoir of the self-watering pots can hold 1 L nutrient solution. The lettuce seedlings were allowed to grow in normal atmospheric pressure in a controlled growth chamber for another 17 d. Only containers with uniform, 27-day-old plants were selected and transferred to the LPPG chambers for the treatments, so plants were 30-days-old at the termination of 3 d studies. Each chamber contained one pot with three seedlings, ($n$ = 1). For the 3 d study, 1 L of nutrient solution was in the reservoir which was sufficient for the duration of the experiment.

Lighting was approximately 240 or 600 μmol·m$^{-2}$·s$^{-1}$ at canopy level inside of the pressure chambers provided by Sylvania 400 W metal halide (M400U) lamps with a 12 h light – 12 h dark phase, maximum–minimum temperature of 26.1 ± 0.6 – 20.0 ± 0.1 °C and maximum–minimum RH of 91.2% ± 3.7% (dark period) – 83.7% ± 2.7% (light pe-

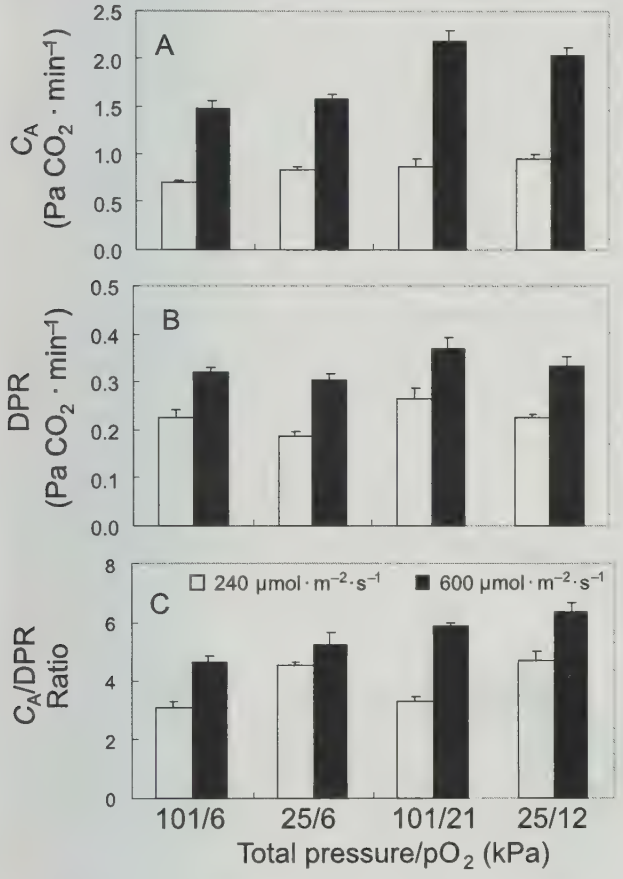



**Fig. 2.** $CO_2$ assimilation ($C_A$), dark-period respiration (DPR), and $C_A$/DPR ratio of lettuce at 101/21, 101/6, 25/12, and 25/6 kPa $pO_2$ at light levels of 240 and 600 μmol·m$^{-2}$·s$^{-1}$. See Table 1 for significance. Bars indicate SE, $n = 3$.

riod). Supplementary $CO_2$ was added during the light cycle to maintain a minimum set point level of 100 Pa $pCO_2$. Without supplementary $CO_2$ during the light period, $CO_2$ levels would fall within several hours to a $CO_2$ compensation point of around 2–4 Pa $CO_2$ (20–40 μL·L$^{-1}$ equivalent at 101 kPa) at 21 kPa $pO_2$.

### Gas exchange, $CO_2$ compensation [$\Gamma(CO_2)$] and $CO_2$ saturation [sat($CO_2$)] points of lettuce plants as affected by total atmospheric pressure, $pO_2$ and light regimes

Plants were exposed to 101 (ambient total pressure)/21 kPa $pO_2$ (normal oxygen at normal pressure) or 25 (low total pressure)/12 kPa $pO_2$. In previous studies, $CO_2$ assimilation ($C_A$) and plant growth and development were comparable between these two treatments (He et al. 2006, 2007). For hypoxic treatments, plants were exposed to 101/6 or 25/6 kPa $pO_2$. Plants were allowed to acclimate for 24 h, and then plant gas exchange, $\Gamma(CO_2)$ and sat($CO_2$) were determined during the light and dark-cycles of the following 2 d. The experiment was terminated at the end of the third day. For the $CO_2$ compensation point study, plants received no supplementary $CO_2$. Subsequently after a couple of days, $CO_2$ deficiency would start to adversely affect plants. Hence, the shorter 3 d study. All plants were of equal size at the initiation and termination of the 3 d experiment, so no growth measurements were recorded.

### $CO_2$ assimilation ($C_A$)

The $C_A$ was measured by rate of $CO_2$ uptake (draw-down) during the light-period cycle (Wheeler 1992; He et al. 2007). The $CO_2$ accumulated in the chambers during the dark-period cycle (without supplementary $CO_2$). To determine $C_A$, slopes of the regression lines of $CO_2$ concentration over time (min) in each chamber were determined during the light cycle. The $pCO_2$ were measured by $CO_2$ sensors within each chamber independently at 1 min intervals during the light cycle. The change of $pCO_2$ in each chamber was related to $C_A$ during the light-period cycle. The minimum $CO_2$ concentration set-point was 100 Pa ($pCO_2$) for all the treatments in ambient and low pressure chambers. When $CO_2$ levels were drawn down (assimilated by plants in the chamber) to 100 Pa, which was equivalent to 1000 μmol·mol$^{-1}$ at 101 kPa total pressure, the system added supplementary gas to maintain the set point level $CO_2$ in each chamber through the remaining duration of the light-period cycle. The rates of $C_A$ were obtained during the first 100 min (10 min after lights were turned on during the light cycle). Hence, at 1 min intervals, there were 100 measurements taken in determining the daily $C_A$ in each chamber (one container per chamber with three seedling plants per container) per treatment. There were three replicated chambers ($n = 3$) per treatment.

### Dark-period respiration (DPR)

The DPR was measured by $CO_2$ accumulation in chambers during the dark period (Wheeler 1992; Richards et al. 2006; He et al. 2007). The $pCO_2$ data were measured by $CO_2$ sensors within each chamber independently at 1 min intervals during the dark period. During the dark-period cycle, no supplementary $CO_2$ was added to chambers and $CO_2$ was allowed to rise without maximum set points. To avoid any residual effects of the light cycle, data during the first 10 min of the dark cycle were not used for calculation. Hence, there were 710 × 1 min interval measurements taken nightly for DPR in each chamber per treatment. DPR was determined by the slopes of the regression equations of accumulated $CO_2$ concentration in each chamber during the dark-period cycle. There were three replicated chambers, $n = 3$.

### Determination of $CO_2$ compensation point [$\Gamma(CO_2)$] and $CO_2$ saturation point [sat($CO_2$)]

During the light period, the $\Gamma(CO_2)$ and sat($CO_2$) of lettuce was measured by $CO_2$ sensors in chambers under total pressures of 101/21 and 25/12 $pO_2$, hypoxic conditions of 101/6 and 25/6 $pO_2$, and light irradiance of 240 or 600 μmol·m$^{-2}$·s$^{-1}$. Plants were acclimated in chambers for 1 d, and $\Gamma(CO_2)$ and sat($CO_2$) measurements taken during days 2 and 3. Data from day 3 are presented. For $\Gamma(CO_2)$, the $pCO_2$ was measured at 1 min intervals during the 240 min period from the initial partial pressure of $CO_2$ at 100 Pa. There were no supplementary $CO_2$ added to chambers during this period, and $pCO_2$ allowed to reach $\Gamma(CO_2)$.

Data were the averages of the last 100 min from the $CO_2$ sensor readings ($n = 3$; total 100 readings per replication).

For sat($CO_2$), measurements were initiated before noon with the initial $pCO_2$ substrate levels at ≅ 400 Pa from natural accumulation during the dark-period. In the afternoon, supplementary $CO_2$ of ≅ 400 Pa was added and additional sat($CO_2$) measurements determined. In preliminary experiments, up to 800 Pa of $CO_2$ was added; at around 250 Pa of $CO_2$, the plateau of the curve was reached, so sat($CO_2$) had been exceeded.

### Statistical analysis

Replication was achieved by repeating treatments under the same conditions over time. Low and ambient total pressure chambers were run concurrently. Chambers were alternated during treatments between low and ambient total pressure at hypoxic and nonhypoxic conditions to avoid any chamber effects. There were no chamber effects. For a given experimental run, all chambers were at either 240 or 600 $\mu mol \cdot m^{-2} \cdot s^{-1}$. All reported data were pooled from repeated independent treatments. There were six chambers, so replications of treatments were run with combinations of total pressure and partial pressure of $pO_2$ and repeated as needed under the same environmental conditions. There were three plants per container and one container per chamber, with each container as a single replicate. There were three replicates per treatment ($n = 3$). ANOVA was also conducted to determine main effects and interactions, with mean separation by ± SE for both gas exchange, $\Gamma(CO_2)$ and sat($CO_2$).

## Results

### $CO_2$ assimilation ($C_A$) and dark-period respiration

Lettuce plants grown under low light had the lowest $C_A$, DPR, and $C_A$/DPR ratio ($P < 0.001$), independent of total pressure and $pO_2$ conditions (Fig. 2). $C_A$ was independent of total pressure, whereas hypobaria generally decreased DPR and increased the $C_A$/DPR ratio (Fig. 2). $C_A$ more than doubled when light was increased from 240 to 600 $\mu mol \cdot m^{-2} \cdot s^{-1}$, regardless of $pO_2$ or total pressure. Under high light there was generally no difference in $C_A$, DPR, or $C_A$/DPR ratio between 101/21 and 25/12 kPa $pO_2$ plants. Lettuce grown at 25/6 kPa $pO_2$ had the lowest DPR (Fig. 2). DPR was the highest at 101/21 kPa $pO_2$ under high light. The $C_A$/DPR ratio was significantly affected ($P < 0.01$) by total pressure, $pO_2$, light irradiance, and the interactions of total pressure × light irradiance ($P < 0.05$), and $pO_2$ × light irradiance ($P < 0.05$) (Table 1; Fig. 2). The $C_A$/DPR ratio was lowest in ambient pressure plants under low light, independent of $pO_2$ (Fig. 2). The $C_A$/DPR ratio was generally greater in hypobaric than ambient pressure plants independent of $pO_2$ and light conditions. The higher $C_A$/DPR ratio suggests a greater efficiency of $C_A$ to DPR with hypobaric plants (Fig. 2). Under high light, the $C_A$/DPR ratio of 101/21 was greater than 101/6 kPa $pO_2$ plants, but under low light, hypoxia had no effect on the $C_A$/DPR of ambient pressure plants. The $C_A$/DPR ratio was greater at ambient than hypoxic levels, regardless of pressure. However, under low light, the $C_A$/DPR ratio was greater under low pressure than ambient, regardless of $pO_2$ levels.

### $CO_2$ compensation point [$\Gamma(CO_2)$]

The $\Gamma(CO_2)$ was independent of total pressure (Table 1; Fig. 3), as was $C_A$. Both hypoxia and high light ($P < 0.01$) caused significant reduction in $\Gamma(CO_2)$, regardless of total pressure (Fig. 3). There was a significant interaction ($P < 0.001$) between $pO_2$ and light irradiance, with hypoxic and low-light plants having the lowest $\Gamma(CO_2)$ (Fig. 3). $CO_2$ was assimilated more rapidly under high light (600 $\mu mol \cdot m^{-2} \cdot s^{-1}$) with a faster draw down to reach $\Gamma(CO_2)$ than low light (240 $\mu mol \cdot m^{-2} \cdot s^{-1}$). There was an interaction ($P < 0.001$) of $pO_2$ and light (Fig. 4) as hypoxic and high light plants reached $\Gamma(CO_2)$ earlier than 12 or 21 $pO_2$ or low-light plants (Fig. 4).

### $CO_2$ saturation point [sat($CO_2$)]

The sat($CO_2$) was significantly ($P < 0.001$) affected by total pressure, $pO_2$, light, and the interaction ($P < 0.01$) of pressure and $pO_2$ (Fig. 5; Table 1). The highest sat($CO_2$) was about 150 Pa $CO_2$ at 101/21 kPa $pO_2$ under high light. The sat($CO_2$) of 101/21 kPa $pO_2$ was reduced to 115 Pa $CO_2$ (–23%) under low light. Low light reduced the sat($CO_2$) independent of total pressure. Hypoxia had no effect on $_{sat}(CO_2)$ of hypobaric plants, whereas hypoxia decreased the $_{sat}(CO_2)$ of ambient-pressure plants (Figs. 5 and 6). The sat($CO_2$) was equally low among low-light plants at 101/6, 25/6, and 25/12 kPa $pO_2$. The sat($CO_2$) was lower in hypobaric than ambient pressure plants independent of $pO_2$ and light.

## Discussion

This is one of the first reports that both $C_A$ and $\Gamma(CO_2)$ are independent of total atmospheric pressure, indicating that hypobaria (25/12 kPa $pO_2$) had no adverse affect on the photosynthetic efficiency of lettuce. Low light and hypoxia greatly depressed $\Gamma(CO_2)$. However the sat($CO_2$), DPR, and CA/DPR ratio were affected by total pressure, hypoxia, and irradiance. Hypoxia decreased the sat($CO_2$) of ambient plants, but had no adverse effect on hypobaric plants. While low light reduced sat($CO_2$), it increased the $\Gamma(CO_2)$ compared with high-light plants. Not only did hypobaric conditions not adversely affect gas exchange, but there are advantages during hypoxic conditions, compared with ambient total pressure plants. These results concur with longer-term studies showing that lettuce can successfully be grown under hypobaria (He et al. 2007).

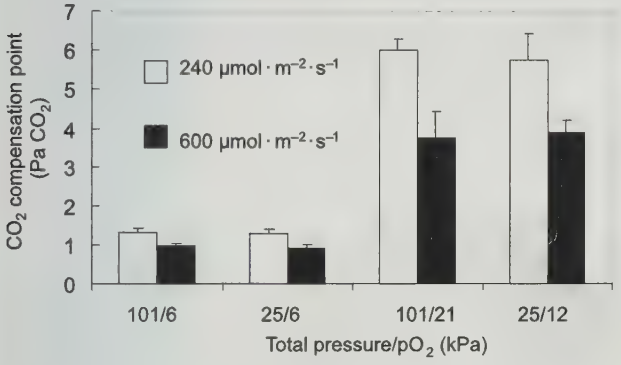
This research further documents that DPR was reduced by hypobaria. The $C_A$/DPR ratio was increased by hypobaria, while it was decreased by hypoxia and low light (Fig. 2; Table 1). The $C_A$/ DPR ratio is an indication of energy available for photosynthesis and growth, including how efficiently carbohydrates are utilized during DPR, and has been used to predict growth performance (Smith et al. 1995). All plants were of equal size at the initiation and termination of the 3 d study. In an earlier study, there was no difference in vegetative growth in 25/12 and 101/21 kPa $pO_2$ plants, and roots were healthy (He et al. 2007). Iwabuchi et al. (1996) reported that there were no differences in $C_A$ and transpiration of spinach at 101/21, 25/21, and 25/10 kPa $pO_2$ during a 31 d study. However, Corey et al. (1996) observed that $C_A$ of lettuce at reduced pressure was

**Table 1.** Effect of total atmospheric pressure (101 or 25 kPa), partial pressure of oxygen (6, 12, or 21 kPa $pO_2$), and light (240 or 600 $\mu mol \cdot m^{-2} \cdot s^{-1}$) on $CO_2$ assimilation ($C_A$), dark-period respiration (DPR), $C_A$ / DPR ratio, $CO_2$ compensation point [$\Gamma(CO_2)$] and saturation point [sat($CO_2$)] of lettuce.

| | $C_A$ | DPR | $C_A$/DPR ratio | CO2 compensation point | $CO_2$ saturation point |
|---|---|---|---|---|---|
| Pressure (P) | NS | * | ** | NS | *** |
| Oxygen (O) | *** | ** | ** | *** | *** |
| Light (L) | *** | *** | *** | *** | *** |
| P x O | NS | NS | NS | NS | ** |
| O x L | ** | NS | * | ** | NS |
| P x L | NS | NS | * | NS | NS |
| P x O x L | NS | NS | NS | NS | NS |

**Note:** NS, non-significant; *, $P < 0.05$ **, $P < 0.01$; ***, $P < 0.001$; $n = 3$.

**Fig. 3.** $CO_2$ compensation point [$\Gamma(CO_2)$] of lettuce at 101 / 21, 101 / 6, 25 / 12, and 25 / 6 kPa $pO_2$ at light levels of 240 and 600 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Data were the averages of the final 100 min of the draw-down. See Table 1 for significance. Bars indicate SE, $n = 3$.




14.6% higher than ambient pressure, and that decreased $pO_2$ resulted in higher $C_A$, regardless of total pressure. In a study of *Arabidopsis* gene expression at low atmospheric pressure, less than one-half of the 200 genes dramatically up or down-regulated by hypobaria were similarly affected by hypoxia, suggesting that the response to hypobaria is unique and more complex than an adaptation to reduced $pO_2$ (hypoxia) inherent to hypobaric environments, i.e., hypobaria does not equal hypoxia (Paul et al. 2004).

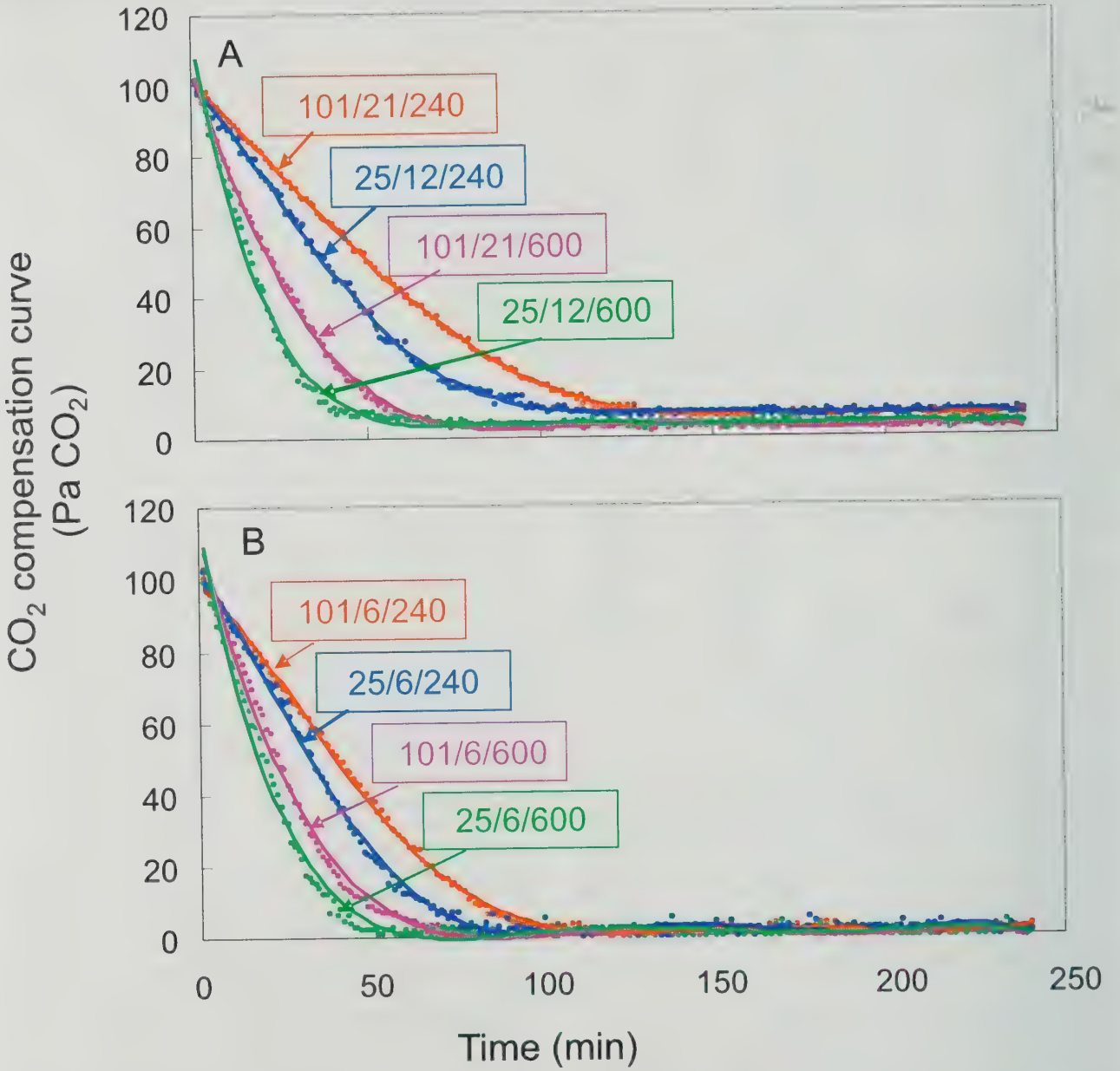
### Variables affecting $\Gamma(CO_2)$

The $\Gamma(CO_2)$ is the $CO_2$ equilibrium between the rate of photosynthesis and leaf respiration and photorespiration (Smith et al. 1976). Factors such as temperature, $pO_2$, and water stress alter the $\Gamma(CO_2)$. Hypoxia was the major factor lowering $\Gamma(CO_2)$ in the LPPG system. At ambient total pressure, $\Gamma(CO_2)$ was about 6 Pa $pCO_2$ ($\sim$60 ppm) at 21 kPa $O_2$ under low-light condition (Fig. 3). The $\Gamma(CO_2)$ of lettuce was in the range of 4 to 5 Pa $pCO_2$ reported for terrestrial plants which fix C directly via the Calvin ($C_3$) cycle (Espie and Colman 1987) However, under hypoxia $\Gamma(CO_2)$ of lettuce was reduced to 1.3 Pa (–78%). A similar pattern was observed under hypobaria (25 kPa). It is likely that the low $\Gamma(CO_2)$ during hypoxia was related to respiration and photorespiration. Typically, $C_3$ plants release less photorespiratory $CO_2$ which leads to a lower $\Gamma(CO_2)$ under hypoxic conditions. Regular cellular respiration may be another factor contributing to a lower $\Gamma(CO_2)$. However, high $pCO_2$ (100 Pa) substrate levels were used in our studies, so photorespiration was not a limiting factor to plants in the LPPG system. Our data reveal that hypoxia lowered DPR of ambient pressure plants, but had little effect on hypobaric plants (Fig. 2; Table 1).

Most respiration in lettuce is the result of maintenance respiration, not growth respiration (Van Iersel 2003). While we are not suggesting that maintenance respiration per se is undesirable for plant growth, hypobaria reduced DPR at 6 kPa $pO_2$ (He et al. 2007). It has been reported that hypobaric storage reduces the respiration of stored fruits and vegetables (Burg 2004). While the mechanism of reduced DPR under hypobaria is not known, there may be growth advantages of hypobaria and higher $pCO_2$ to limit respiration losses, particularly under hypoxic conditions (Corey et al. 1996). Under ambient pressure, decreased DPR at elevated $CO_2$ is likely the result of direct inhibition of the activity of two mitochondrial enzymes: succinate dehydrogenase and cytochrome *c* oxidase; elevated $pCO_2$ leads to higher $C_A$ in a number of plant species, increasing the demand for reductant to be used in carbon fixation (Griffin et al. 2001); they reported that despite an increase in mitochondrial number in a number of plant species under elevated $CO_2$, DPR decreased, suggesting the rate of ATP production via oxidative phosphorylation decreased. Low $pO_2$ also reduces the competition of $CO_2$ for RuBisCO, which lowers the $\Gamma(CO_2)$.

Photorespiration is nearly eliminated by elevating $CO_2$ above 100 Pa (as was done in our study), hence electron transport becomes the limiting factor for $CO_2$ fixation (Farquhar et al. 1980). Even under warm temperatures, photorespiration is reduced by raising $pCO_2$, with electron transport becoming the rate-limiting factor in $CO_2$ fixation (Frantz et al. 2002, 2004). Inhibition of photorespiration has been suggested as a reason for enhanced $C_A$ of hypobaric plants (Iwabuchi et al. 1995, 1996; Corey et al. 1996, 2002; Goto et al. 1996). Richards et al. (2006) suggested that differences in photosynthetic drawdown rates with *Arabidopsis* were largely due to $pO_2$ effects on photorespiration and not due to hypobaria. In a 24 h study with *Arabidopsis* seedlings under high $pCO_2$, hypobaria had no significant effect on the differential expression of five key photorespiration enzymes, including RuBisCO; there was less than two-fold changes in regulation of the targeted genes, suggesting no altered regu-

**Fig. 4.** $CO_2$ compensation point [$\Gamma(CO_2)$] curves of hypobaric (25 kPa) and ambient (101 kPa) total pressure lettuce plants at 240 and 600 $\mu mol \cdot m^{-2} \cdot s^{-1}$, under (A) nonhypoxic (21 or 12 kPa $pO_2$) or (B) hypoxic (6 kPa $pO_2$) conditions. The $pCO_2$ was measured at 1 min intervals during 240 min draw-down period from the initial concentration of $CO_2$ at 100 Pa.




lation within the photorespiratory pathway to hypobaria (Richards et al. 2006).

Heichel (1971) reported that glycolate formation and metabolism during photorespiration had been shown to be enhanced not only with decreased $CO_2$ concentrations and increased $O_2$, but also by high light. We observed that high irradiance decreased $\Gamma(CO_2)$ under ambient and hypobaric pressure (Fig. 3). This is in conflict with open field environments where $\Gamma(CO_2)$ is higher rather than lower under high light conditions. Under open field conditions, high irradiance leads to high temperature and water stress, causing stomata to partially or fully close. Subsequently $pCO_2$ substrate is reduced in the leaf mesophyl. However, in our semi-closed, LPPG system, constant temperature (26.1% ± 0.6 °C) and humidity (83.7% ± 2.7%) were maintained during the light-period. Consequently, lettuce in the LPPG system had greater $C_A$ and assimilated $CO_2$ more rapidly under high than lower light, since the normal field constraints of temperature and water stress were not factors (Fig. 4).

Smith et al. (1976) reported that increasing light intensities did not affect $\Gamma(CO_2)$ of soybean grown at 500 and then placed under 800 $\mu mol \cdot m^{-2} \cdot s^{-1}$ supplemental light. In

**Fig. 5.** $CO_2$ saturation point [sat($CO_2$)] of lettuce at 101/21, 101/6, 25/12, and 25/6 kPa $pO_2$ at light levels of 240 and 600 μmol·m$^{-2}$·s$^{-1}$. Data were the averages of $pCO_2$, beyond which the $CO_2$ response curve reached a plateau. See Table 1 for significance. Bars indicate SE, $n$ = 3.

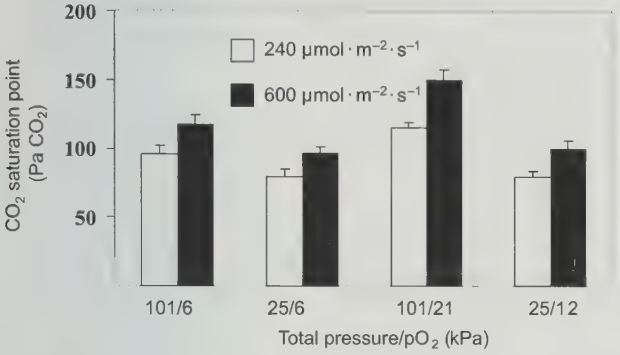
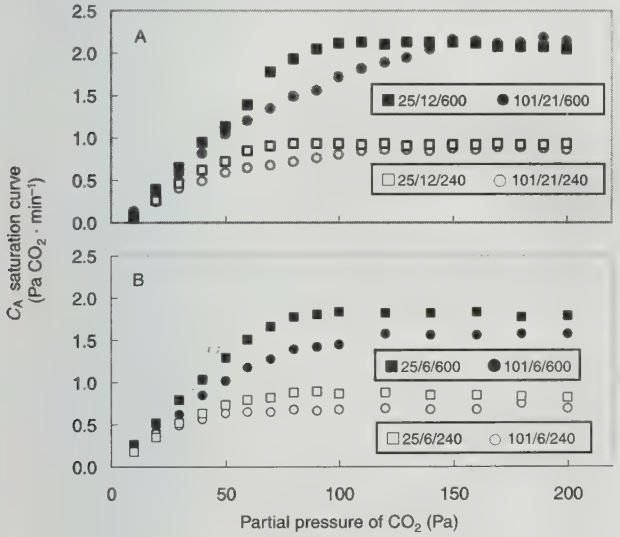



**Fig. 6.** $CO_2$ saturation point [sat($CO_2$)] curves of hypobaric (25 kPa) and ambient (101 kPa) total pressure lettuce plants at 240 and 600 μmol·m$^{-2}$·s$^{-1}$, under (A) nonhypoxic (21 or 12 kPa $pO_2$) or hypoxic (6 kPa $pO_2$) conditions. Data was collected from 0 to 200 kPa $pCO_2$, and the plateau of sat($CO_2$) determined.




our experiment with lettuce, decreasing light from 600 to 240 μmol·m$^{-2}$·s$^{-1}$ increased $\Gamma(CO_2)$. We observed that $C_A$ increased proportionally to light irradiance, indicating that light was not saturated at 600 μmol·m$^{-2}$·s$^{-1}$ in our LPPG system. In a previous study, light saturation of lettuce was around 800 μmol·m$^{-2}$·s$^{-1}$ (He et al. 2006). In regard to future controlled-environment agriculture (CEA) in lunar and Martian habitats, where energy usage is critical, crops will be grown at lower, not higher irradiance levels.

Hypobaric plants reached $\Gamma(CO_2)$ sooner than ambient total pressure plants, regardless of $pO_2$ and light irradiance (Fig. 4). Our data suggests that hypobaric plants use $CO_2$ more efficiency than ambient pressure plants. This is likely due to the hypobaric effect on gaseous diffusion, which increases with the loss of leaf boundary layer resistance (Rygalov et al. 2004). Hypobaria affects gas diffusion rates and surface boundary layers and increases convective transfer capabilities and water evaporation rates (Goto et al. 1996; Richards et al. 2006). Our data agree with Richards et al. (2006), who reported that $C_A$ of suboptimal $pCO_2$ (40 Pa) plants was greater at hypobaric than ambient pressure, but when $pCO_2$ was in a nonlimiting range (70 to 100 Pa), $C_A$ was insensitive to hypobaria in a short-term, 16 h study of *Arabidopsis*.

### Variables affecting sat($CO_2$)

The sat($CO_2$) is not a factor in growing crops in the open fields because sufficiently high $CO_2$ substrate levels are not reached. However, it is very important parameter when growing plants in CEA from terrestrial greenhouses to more elaborate NASA life support systems, where $CO_2$ can be maintained at sat($CO_2$) for maximum growth. The potential range of environmental conditions in an ALS include total gas pressures as low as 54 kPa (8 psi), and $CO_2$ concentrations as high as 700 Pa (7000 μmol·mol$^{-1}$), some 19 times greater than on Earth (NASA 1998, 2002, 2004), and in great excess of the range that researchers use to study global change (Amthor 1991). Furthermore, the Martian atmosphere is 95% $CO_2$, which could be used as a readily available substrate. We observed that hypobaria reduced sat($CO_2$) regardless of $pO_2$. However, while hypoxia decreased sat($CO_2$) of ambient pressure plants, hypobaric plants were not affected (Fig. 5). Low light reduced sat($CO_2$), as plants reached sat($CO_2$) earlier than plants under high light (Fig. 6); this is due to light irradiance below light saturation.

Supraoptimal $CO_2$ effects on soybean at ambient pressure were reported (Wheeler et al. 1993); stomatal conductance decreased at 100 or 200 Pa $CO_2$, but increased above 200 Pa $CO_2$; however, seed yield and total biomass were optimal at 100 Pa. There are advantages of exposing lettuce to higher $CO_2$ conditions. During the light-period cycle, the supplementary set point of $pCO_2$ was 100 Pa, which is much greater than earth ambient $pCO_2$ (36 Pa). The higher $pCO_2$ is in line with conditions most likely to occur under lunar and Martian CEA systems (Corey et al. 2002; Paul and Ferl 2006).

Increased $pCO_2$ is reported to enhance anion channel activity, which are proposed to mediate efflux of osmoregulatory anions ($Cl^-$ and malate$^{-2}$) from *Vicia faba* guard cells during stomatal closure (Schroeder and Hagiwara 1989). Negi et al. (2008) isolated the *Arabidopsis* gene, *SLAC1*, which mediates $CO_2$ sensitivity in regulation of plant gas exchanges. We did not find any differences in guard cells or stomatal anatomy in the leaves of hypobaric and ambient pressure plants at 100 Pa $pCO_2$ (data not shown).

In summary, this study shows that hypobaria (25 kPa) caused no adverse affects on plant gas exchange, and may be advantageous in CEA systems experiencing hypoxia.

## Acknowledgements

This research was supported in part by the National Aeronautics and Space administration (NASA) through Grants No.NAG-9-1067, Plant Growth and Metabolism at Sub-Ambient Atmospheric Pressures; and No. NAJ04HF31G,

Plant Growth at Sub-Ambient Atmospheric Pressures with Control of the Partial Pressures of Constituent Gases; and the Texas Agricultural Experiment Station.

## References


Amthor, J.S. 1991. Respiration in a future, higher-$CO_2$ world. Plant Cell Environ. **14**: 13–20. doi:10.1111/j.1365-3040.1991.tb01367.x.

Andre, M., and Massimino, D. 1992. Growth of plants at reduced pressures: experiments in wheat- technological advantages and constraints. Adv. Space Res. **12**: 97–106. doi:10.1016/0273-1177(92)90015-P. PMID:11537084.

Bugbee, B.G., and Salisbury, F.B. 1989*a*. Current and potential productivity of wheat for a controlled environment life support system. Adv. Space Res. **9**: 5–15. doi:10.1016/0273-1177(89)90024-0. PMID:11537390.

Bugbee, B.G., and Salisbury, F.B. 1989b. Controlled environment crop production: hydroponic vs. lunar regolith. *In* Lunar base agriculture: soils for plant growth. *Edited by* D.W. Ming and D.L. Henninger. American Society of Agronomy, Madison, Wisc. pp. 107–129.

Burg, S.P. 2004. Postharvest physiology and hypobaric storage of fresh produce. CAB International, Wallingford, Oxfordshire, UK.

Corey, K.A., Bates, M.E., and Adams, S.L. 1996. Carbon dioxide exchange of lettuce plants under hypobaric conditions. Adv. Space Res. **18**: 301–308. doi:10.1016/0273-1177(95)00820-5. PMID:11538976.

Corey, K.A., Barta, D.J., and Wheeler, R.M. 2002. Toward Martian agriculture: responses of plants to hypobaria. Life Support Biosph. Sci. **8**: 103–114. PMID:11987302.

Davies, F.T., Jr., Calderón, C.M., Huaman, Z., and Gómez, R. 2005. Influence of a flavonoid (formononetin) on mycorrhizal activity and potato crop productivity in the highlands of Peru. Sci. Hortic. **106**: 318–329. doi:10.1016/j.scienta.2005.04.013.

Drew, M.C. 1997. Oxygen deficiency and root metabolism: injury and acclimation under hypoxia and anoxia. Annu. Rev. Plant Physiol. Plant Mol. Biol. **48**: 223–250. doi:10.1146/annurev.arplant.48.1.223. PMID:15012263.

Espie, G.S., and Colman, B. 1987. The effect of pH, $O_2$ and temperature on the $CO_2$ compensation point of isolated *Asparagus* mesophyll cells. Plant Physiol. **83**: 113–117. doi:10.1104/pp.83.1.113. PMID:16665183.

Farquhar, G.D., von Caemmeerer, S., and Berry, J.A. 1980. A biochemical model of photosynthetic assimilation in leaves of $C_3$ species. Planta, **149**: 78–90. doi:10.1007/BF00386231.

Frantz, J., Ritchie, G., Cometti, N., Robinson, J., and Bugbee, B. 2002. Exploring the limits of crop productivity: quantum yield, radiation capture, and carbon use efficiency of lettuce in a high light, temperature, and $CO_2$ environment. [Online]. Utah State University, Logan, Utah, US. Available from www.usu.edu/cpl/research_lettuce2.htm.

Frantz, J.M., Ritchie, G., Cometti, N.N., Robinson, J., and Bugbee, B. 2004. Exploring the limits of crop productivity: beyond the limits of tipburn in lettuce. J. Am. Soc. Hortic. Sci. **129**: 331–338. PMID:15776542.

Gale, J. 1972. Availability of carbon dioxide for photosynthesis at high altitudes: theoretical considerations. Ecology, **53**: 494–497. doi:10.2307/1934239.

Gale, J. 1973. Experimental evidence for the effect of barometric pressure on photosynthesis and transpiration. *In* Plant response to climatic factors. Proceedings of the Uppsala Symposium, UNESCO, Paris, France. pp. 289-294.

Goto, E., Ohta, H., Iwabuchi, K., and Takakura, T. 1996. Measurement of net photosynthetic and transpiration rates of spinach and maize plants under hypobaric conditions. J. Agric. Meteorol. **52**: 117–123.

Griffin, K.L., Anderson, O.R., Gastrich, M.D., Lewis, J.D., Lin, G., Schuster, W., Seemann, J.R., Tissue, D.T., Turnbull, M.H., and Whitehead, D. 2001. Plant growth in elevated CO2 alters mitochondrial number and chloroplast fine structure. Proc. Natl. Acad. Sci. U.S.A. **98**: 2473–2478. doi:10.1073/pnas.041620898. PMID:11226263.

He, C., Davies, F.T., Jr., Lacey, R.E., Drew, M.C., and Brown, D.L. 2003. Effect of hypobaric conditions on ethylene evolution and growth of lettuce and wheat. J. Plant Physiol. **160**(11): 1341–1350. doi:10.1078/0176-1617-01106. PMID:14658387.

He, C., Davies, F.T., Jr., and Lacey, R.E. 2006. Hypobaric conditions effect gas exchange, ethylene evolution and growth of lettuce for advanced life support systems (ALS). Habitation (Elmsford), **11**: 49–61. doi:10.3727/154296606779507088.

He, C., Davies, F.T., Jr., and Lacey, R.E. 2007. Separating the effects of hypobaria and hypoxia on lettuce: growth and gas exchange. Physiol. Plant. **131**: 226–240. PMID:18251894.

Heichel, G.H. 1971. Response of respiration of tobacco leaves in light and darkness and the CO2 compensation concentration to prior illumination and oxygen. Plant Physiol. **48**: 178–182. doi:10.1104/pp.48.2.178. PMID:16657758.

Hough, A., and Wetzel, R. 1978. Photorespiration and $CO_2$ compensation point in Najars flexilis. Limnol. Oceanogr. **23**(4): 719–724.

Iwabuchi, K., Goto, E., and Takakura, T. 1995. Effect of $O_2$ pressure under low air pressure on net photosynthetic rate of spinach. Acta Hortic. **399**: 101–106.

Iwabuchi, K., Goto, E., and Takakura, T. 1996. Germination and growth of spinach under hypobaric conditions. Environ. Control Biol. **34**: 169–178.

Musgrave, M.E., Gerth, W.A., Scheld, H.W., and Strain, B.R. 1988. Growth and mitochondrial respiration of mungbeans (*Phaseolus aureus* Roxb.) germinated at low pressure. Plant Physiol. **86**: 19–22. doi:10.1104/pp.86.1.19. PMID:11538232.

NASA. 1998. Advanced life support program. Requirements, definition and design considerations. NASA Headquarters, Houston, Texas. Publication Johnson Space Center No. 38571.

NASA. 2002. Advanced life support baseline values and assumptions document, Lyndon B. Johnson Space Center, NASA Headquarters, Houston, Texas. Publication Johnson Space Center No. 47804.

NASA. 2004. Advanced life support program. Bounding the design space for spacecraft internal atmosphere pressure and composition. Office of Biological and Physical Research, NASA Headquarters, Houston, Tex.

Negi, J., Matasuda, O., Nagasawa, T., Oba, Y., Takahashi, H., Kawai, M., Uchimiya, H., Hashimoto, M., and Iba, K. 2008. $CO_2$ regulator SLAC1 and its homologues are essential for anion homoeostasis in plant cells. Nature (London), **452**: 483–486. doi:10.1038/nature06720. PMID:18305482.

Paul, A.L., and Ferl, R.F. 2006. The biology of low atmospheric pressure – implications for exploration mission design and advanced life support. Gravit. Space Biol. Bull. **19**: 3–18.

Paul, A.L., Schuerger, A.C., Popp, M.P., Richards, J.T., Manak, M.S., and Ferl, R.J. 2004. Hypobaric biology: Arabidopsis gene expression at low atmospheric pressure. Plant Physiol. **134**: 215–223. doi:10.1104/pp.103.032607. PMID:14701916.

Richards, J.T., Corey, K.A., Paul, A.L., Ferl, R.J., Wheeler, R.M., and Schuerger, A.C. 2006. Exposure of *Arabidopsis thaliana* to hypobaric environments: implications for low-pressure bioregenerative life support systems for human exploration missions and

terraforming on Mars. Astrobiology, **6**: 851–866. doi:10.1089/ast.2006.6.851. PMID:17155885.

Rygalov, V.Y., Fowler, P.A., Wheeler, R.M., and Bucklin, R.A. 2004. Water cycle and its management for plant habitats at reduced pressures. Habitation (Elmsford), **10**(1): 49–59. doi:10.3727/154296604774808865. PMID:15880909.

Schroeder, J.I., and Hagiwara, S. 1989. Cytosolic calcium regulates ion channels in the plasma membrane of *Vicia faba* guard cells. Nature (London), **338**: 427–430. doi:10.1038/338427a0.

Schwartzkopf, S.H., and Mancinelli, R.L. 1991. Germination and growth of wheat in simulated Martian atmospheres. Acta Astronaut. **25**: 245–247. doi:10.1016/0094-5765(91)90078-J. PMID:11537561.

Smith, E.W., Tolbert, N.E., and Ku, H.S. 1976. Variables affecting the $CO_2$ compensation point. Plant Physiol. **58**: 143–146. doi:10.1104/pp.58.2.143. PMID:16659635.

Smith, B.N., Lytle, C.M., and Hansen, L.D. 1995. Predicting plant growth rates from dark respiration rates: an experimental approach. *In* Wildland Shrub and Arid Land Restoration Symposium: Proceedings. *Edited by* B.A. Roundy, E.D.McArthur, J.S. Haley, and D.K. Mann. Oct 19–21, 1993, Las Vegas, Nevada. Gen Tech Rep. INT-GTR-315. USDA For. Serv., Intermountain Research Station, Ogden, Utah, US.

Spanarkel, R., and Drew, M.C. 2002. Germination and growth of lettuce (*Lactuca sativa*) at low atmospheric pressure. Physiol. Plant. **116**: 468–477. doi:10.1034/j.1399-3054.2002.1160405.x. PMID:12583399.

Tolbert, N.E., Benker, C., and Beck, E. 1995. The oxygen and carbon dioxide compensation points of $C_3$ plants: possible role in regulating atmospheric oxygen. Proc. Natl. Acad. Sci. U.S.A. **92**: 11230–11233. doi:10.1073/pnas.92.24.11230. PMID:11607591.

Van Iersel, M.W. 2003. Carbon use efficiency depends on growth respiration, maintenance respiration, and relative growth rate. A case study with lettuce. Plant Cell Environ. **26**: 1441–1449. doi:10.1046/j.0016-8025.2003.01067.x.

von Caemmerer, S., and Faquhar, G.D. 1981. Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. Planta, **153**: 376–387. doi:10.1007/BF00384257.

Wheeler, R.M. 1992. Gas-exchange measurements using a large, closed plant growth chamber. HortScience, **27**: 777–780. PMID:11537623.

Wheeler, R.M., Mackowiak, C.L., Siegriest, L.M., and Sager, J.C. 1993. Supraoptimal carbon dioxide effects on growth of soybean [*Glycine max* (L.) Merr.]. J. Plant Physiol. **142**: 173–178. PMID:11538190.

Wheeler, R.M., Mackowiak, C.L., Sager, J.C., Berry, W.A., and Yorio, N.C. 1994. Lettuce growth and gas exchange in a closed, controlled environment. J. Am. Soc. Hortic. Sci. **119**: 610–615. PMID:11538197.

Wheeler, R.M., Stutte, G.W., Subbarao, G.V., and Yorio, N.C. 2001. Plant growth and human life support for space travel. *In* Handbook of plant and crop physiology, 2nd ed.. *Edited by* M. Pessarakli. Marcel Dekker, Inc., New York, N.Y.

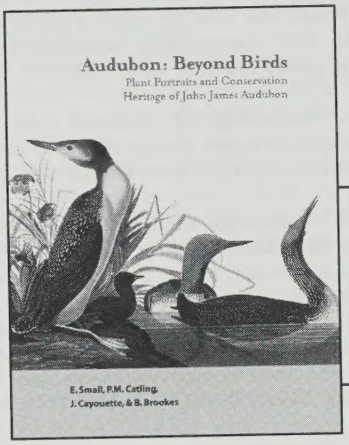
NRC·CNRC
From Discovery to Innovation...
De la découverte à l'innovation...

La Science à l'œuvre pour le Canada
at work for Canada

New release / Nouvelle parution

# Audubon: Beyond Birds

## Plant Portraits and Conservation Heritage of John James Audubon




E. Small, Paul M. Catling, J. Cayouette, and B. Brookes

270 Pages 2009
Hardcover / Édition reliée
ISBN-13 978-0-660-19894-1; NRC / CNRC : 49731
$49.95 CAN; Other countries $49.95 US
49,95 $CAN; À l'étranger : 49,95 $US

John James Audubon was one of the world's greatest painters of wildlife. He is best known for The Birds of America, a gigantic four-volume set of 435 paintings that to this day remains one of the largest books ever produced, the most expensive book ever sold at auction and an artistic achievement almost without parallel. Audubon also prepared a similar three-volume set of 150 outstanding paintings of North American mammals. In the last 2 centuries, dozens of books have been produced, either stressing Audubon's work as pure art or documenting the animals that were painted. The present volume is unique in emphasizing the plants that Audubon frequently illustrated along with his animals. Superb, full-color reproductions are shown of more than 100 of Audubon's best paintings, chosen for their excellent portrayal of plants. Each magnificent full-page plate is accompanied by information on the animals (mostly birds), the painting and (most particularly) the plants. An introductory, extensively illustrated chapter details Audubon's life and career — a fascinating story of heroic achievements in the face of great obstacles. A second chapter deals with Audubon's conservation legacy, a topic of considerable importance to the world's ongoing crises related to loss of biodiversity, degradation of the environment and global warming. This book will appeal to everyone who appreciates the stunning beauty of nature and the intriguing life stories of wild plants and animals.

Jean Jacques Audubon était l'un des plus grands peintres animaliers du monde. Il est surtout connu pour Les oiseaux d'Amérique, oeuvre gigantesque en quatre volumes qui comprend 435 planches et qui demeure à ce jour un des plus grands livres jamais créés, le livre le plus cher jamais vendu dans un encan et une oeuvre artistique hors du commun. Audubon a également créé une série similaire de trois volumes comprenant 150 remarquables planches représentant des mammifères d'Amérique du Nord. Au cours des deux derniers siècles, des dizaines de livres ont été publiés, soit pour souligner la nature purement artistique de l'oeuvre d'Audubon, soit pour fournir de la documentation sur les animaux qu'il a peints. Le présent volume est unique en ce sens qu'il présente les plantes qu'Audubon illustrait fréquemment avec ses animaux. Il comprend des reproductions en couleurs d'une centaine des meilleurs tableaux peints par Audubon, choisis pour la qualité de la représentation des plantes qu'on y voit. Chacune des magnifiques planches hors-texte est accompagnée d'information sur les animaux (principalement des oiseaux), sur les tableaux et surtout sur les plantes. L'introduction, abondamment illustrée, relate la vie et la carrière d'Audubon – et l'histoire fascinante d'une oeuvre héroïque réalisée en dépit d'immenses obstacles. Dans un autre chapitre, on aborde la question de l'héritage laissé par Audubon en matière de conservation, un sujet d'une importance considérable dans le contexte des crises que connaît la planète actuellement en matière de perte de biodiversité, de dégradation de l'environnement et de réchauffement climatique. Ce livre plaira à tous ceux qui sont éblouis par la remarquable beauté de la nature et par la fascinante histoire des plantes et des animaux sauvages.

### To order books / Pour commander

**Order Office / Bureau des commandes**
NRC Research Press / Presses scientifiques du CNRC, National Research Council / Conseil national de recherches
Ottawa, ON K1A 0R6, Canada. Telephone / Téléphone : 613-990-2254; Fax / Télécopieur : 613-952-7656
E-mail / Courriel : pubs@nrc-cnrc.gc.ca; Order online / Commandes en ligne : http://pubs.nrc-cnrc.gc.ca

**Shipping and handling extra.**
Canadian customers add GST. Prepayment required unless order is accompanied by a purchase order number. Cheques or money orders should be made payable to: "**Receiver General for Canada, Credit NRC**". For all orders outside Canada, rates are in U.S. dollars. All sales are final.

**Frais d'expédition et de manutention en sus.**
Clients du Canada, ajoutez la TPS. Les commandes doivent être payées d'avance à moins qu'elles ne soient accompagnées d'un numéro de bon de commande. Les chèques ou mandats doivent être libellés à l'ordre du « **Receveur général du Canada, crédit du CNRC** ». Pour les commandes hors-Canada, les tarifs sont en dollars américains. Vente ferme.

01/09/2008

## Ecology — Écologie

ARTICLE — ARTICLE

**Hollydawn Murray, Karen Thompson, and Sheila M. Macfie** — **702–711** — Site- and species-specific patterns of metal bioavailability in edible plants

## Structure and development — Structure et développement

MINIREVIEW — MINISYNTHÈSE

**Tyler J. Donner and Enrico Scarpella** — **678–684** — Auxin-transport-dependent leaf vein formation


JF
BCM
REVIEW

National Research Council Canada
Ottawa, Canada K1A 0R6

Conseil national de recherches Canada
Ottawa, Canada K1A 0R6

Postage paid at Ottawa
Publications mail
Registration No. 40062591

Port payé à Ottawa
Poste-publication
Enregistrement n° 40062591

USPS periodical postage paid at Plattsburgh, NY 12901, USA

# BOTANY

Volume 87, Number 7, July 2009

# BOTANIQUE

Volume 87, numéro 7, juillet 2009

## Special Issue celebrating the 50th Anniversary of the Canadian Society of Plant Physiologists (CSPP)

K. Peter Pauls — v–vii — Introduction to the Special Issue / Introduction du numéro spécial

Barry Shelp and R. Larry Peterson — ix–xi — Introduction to "Nitrate uptake by plant roots" / Introduction pour « Nitrate uptake by plant roots »

### Cell and molecular biology

MINIREVIEW

Kimberley Chin, Wolfgang Moeder, and Keiko Yoshioka — 668–677 — Biological roles of cyclic-nucleotide-gated ion channels in plants: What we know and don't know about this 20 member ion channel family

### Physiology and biochemistry

MINIREVIEW

Anthony D.M. Glass — 659–667 — Nitrate uptake by plant roots

REVIEW

Constance Nozzolillo — 685–690 — "Genealogy" of plant physiology research in Canada and the founding of the Canadian Society of Plant Physiologists

ARTICLES

Man-Ho Oh, Joan L. Huber, Wei Shen, Gurdeep S. Athwal, Xia Wu, and Steven C. Huber — 691–701 — Overexpression of a directed mutant of 14-3-3ω in *Arabidopsis* leaves affects phosphorylation and protein content of nitrate reductase

Chuanjiu He, Fred T. Davies Jr., and Ronald E. Lacey — 712–721 — Hypobaria, hypoxia, and light affect gas exchange and the $CO_2$ compensation and saturation points of lettuce (*Lactuca sativa*)

## Numéro spécial du 50e anniversaire de la Société canadienne de physiologie végétale (SCPV)

Introduction to the Special Issue / Introduction du numéro spécial

Introduction to "Nitrate uptake by plant roots" / Introduction pour « Nitrate uptake by plant roots »

### Biologie cellulaire et moléculaire

MINISYNTHÈSE

Biological roles of cyclic-nucleotide-gated ion channels in plants: What we know and don't know about this 20 member ion channel family

### Physiologie et biochimie

MINISYNTHÈSE

Nitrate uptake by plant roots

SYNTHÈSE

"Genealogy" of plant physiology research in Canada and the founding of the Canadian Society of Plant Physiologists

ARTICLES

Overexpression of a directed mutant of 14-3-3ω in *Arabidopsis* leaves affects phosphorylation and protein content of nitrate reductase

Hypobaria, hypoxia, and light affect gas exchange and the $CO_2$ compensation and saturation points of lettuce (*Lactuca sativa*)

*Continued on inside back cover / Suite au verso*

**Front cover:** Epifluorescence micrograph showing a stomatal complex and thick cuticle of a *Haworthia attenuata*leaf embedded in LR White resin, sectioned, and stained with acid fuchsin. Colour modified. Image supplied by Dr. R. Larry Peterson, University of Guelph.

**Page couverture :** Micrographie par épifluorescence montrant l'appareil stomatique et la cuticule épaisse d'une feuille de *Haworthia attenuata* incluse dans une résine LR White, sectionnée et colorée à la fuchsine acide. Couleur modifiée. Image fournie par R. Larry Peterson, Ph.D., Université de Guelph.

Canada

Printed in Canada by / Imprimé au Canada par
NRC Reprographic Services / Service de reprographie CNRC
Page production by / Mise en page par
NRC Research Press / Presses scientifiques du CNRC

1916-2790(200907)87:7;1-N